Epidemiology of HBV Infection in Singapore*

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Geography, climate and population

Singapore, consisting of one main island and several islets, is situated at the southern tip of the Malay Peninsula. It has an area of 584 square kilometres with a tropical climate (temperature: mean maximum, 31°C; mean minimum, 24°C; mean relative humidity, 74 per cent) and annual rainfall of 228 centimetres. The population of 2.3 million is relatively young (mean age, 19.7 years) and is made up of three main ethnic groups: Chinese (76%), Malays (15%) and Indians (7%).

Problems of HBV infection

Hepatitis B virus (HBV) infection is endemic in Singapore. The prevalence of some of the known HBV-related diseases is listed in Table 1.

Table 1 HBV related problems in Singapore

Carriers	overall rate 5%; 1.2×10^5 carriers
Acute hepatitis	24 - 46 per 10^5 per year; 46% HBV
Chronic hepatitis/cirrhosis	400 - 500 discharges per year
Primary liver cancer	male – 28.7 per 10^5 per year
	female – 7.4 per 10^5 per year

Table 2 HBsAg carrier rates (%) among different ethnic groups in Singapore

	Blood donors (Males)	Pregnant women
Chinese	13.6	6.2
Malay	7.3	2.3
Indian	6.0	0.6

HBsAg carriers

The HBsAg carrier rate is highest among the Chinese, followed by the Malays and then the Indians (Table 2).[1,2] This differential rate is seen both in males as well as in females, although males have a higher rate within each ethnic group.

Age specific prevalence of HBV markers

A total of 569 normal adults (voluntary screening programme) and 272 children aged between one and 12 years, who had been admitted to a major paediatric ward for illnesses not associated with the liver, were screened for HBsAg, anti-HBs and anti-HBc markers.[3]

The frequency of HBsAg (Fig. 1) was highest among the paediatric age group (12%), followed by a drop in the teenage group, and a slow rise subsequently peaking in the 40-49-year age group (8.5%). Care had to be exercised in the interpretation of the 13-19 and > 50 age groups because of the relatively small number of subjects within these groups. On the other hand, the frequency of anti-HBs and anti-HBc (Fig. 1) showed a progressive rise with age reaching about 50 per cent in the 40-49-year age group and 100 per cent in the > 60-year group. The data suggested that HBV infection in Singapore starts early in life, giving rise to particularly high carrier rates, and that HBV infection continues into adult life with a progressive acquisition of HBV markers with age. The

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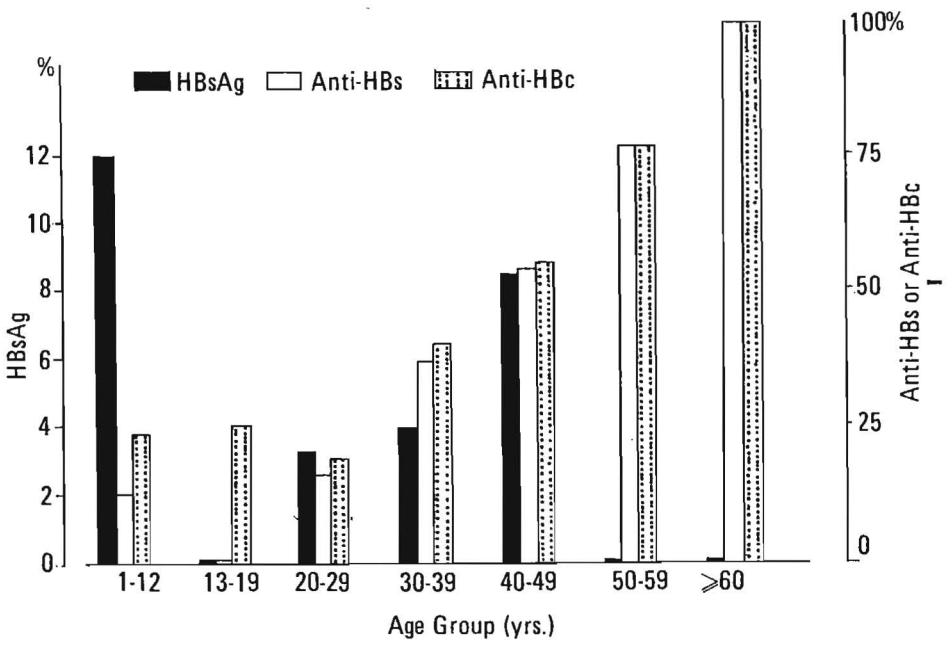

Fig. 1 Age specific prevalence of HBV markers in Singapore

data also suggested that the population is constantly being exposed to HBV and that most of the infections are subclinical.

Perinatal transmission

A study of maternal-infant transmission of HBV was conducted in Singapore between June 1980 and June 1982.[4] A total of 58 HBsAg carrier mothers were studied at delivery from whom 56 infants were followed for one year or longer. Overall HBV transmission occurred in 27 out of 56 (48%). Transmission in the majority of cases was perinatal (84%); intrauterine infection and infection after the perinatal period each accounted for 8 per cent of the cases. The perinatal transmission rate was higher among Chinese (49%) than non-Chinese (29%).

There was a very strong correlation between HBV transmission and the HBeAg status of the mother ($p = 1.85 \times 10^{-9}$; odd ratio = 68) and to a lesser extent with high titre HBsAg (CIE positive; $p = 0.002$; odd ratio = 6.4). A strong negative correlation was seen between transmission and anti-HBe ($p = 8 \times 10^{-7}$; odd ratio = 0.04).

Of the 27 HBV transmissions, 19 infants were still HBsAg positive at one year of age, seven lost their antigenaemia status and acquired anti-HBs and one developed HBsAg after one year.

It could be calculated that with a carrier rate of 4.4 per cent among pregnant mothers and a stable infant HBsAg carrier rate of 34 per cent (19/56) from these carrier mothers, the contribution of maternal-infant HBV transmission to the HBsAg carrier rate of one-year-old children would be 1.5 per cent (34% of 4.4%). The HBsAg carrier rate of children between one month and one year of age admitted to a major paediatric unit for illnesses other than those associated with the liver was 6.5 per cent.[3] If bias due to major illnesses in these children could be ignored, it could be calculated that perinatal transmission accounted for 23 per cent ($\frac{1.5}{6.5} \times 100\%$) of the carrier rate at one year; therefore, about 77 per cent of the carriers must have been infected horizontally, most likely at this age to be infected by members of the family other than the mother.

Acute viral hepatitis

The prevalence of acute viral hepatitis is between 24 and 46 per 100,000 per year and HBV is responsible for about half of these infections.[5] The attack rate of hepatitis due to HBV appears to be highest among Indians followed by Chinese and Malays (Table 3).[6]

HBV and liver diseases

The frequency of HBsAg was significantly higher among patients with primary liver cancer (64%), chronic hepatitis (63%) and non-alcoholic cirrhosis (32%) compared with the normal population (5%; Table 4). On the other hand, the frequency of HBsAg among patients with alcoholic cirrhosis was not significantly elevated. Similarly, the frequency of anti-HBc was significantly higher in cases of primary liver cancer and chronic hepatitis compared with the normal population (Table 5).

Primary liver cancer

Patients with primary liver can-

Table 3 HBV infection, carrier status and primary liver cancer

	*1° liver cancer		HBsAg carriers		Perinatal transmission	**Acute HBV hepatitis
	M	F	M	F		
Chinese	29.8	7.5	13.6	6.2	45%	10.7
Malay	14.4	4.7	7.3	2.3	29%	4.8
Indian	12.9	3.2	6.0	0.6		14.4

* Age standardised rate per 10^5 per year
** Morbidity rate per 10^5 population

Table 4 Prevalence of HBsAg in patients with liver diseases and normal controls in Singapore

	No. tested	No. positive
Primary liver cancer	187	119 (64)
Chronic hepatitis	48	30 (63)
Non-alcoholic cirrhosis	87	28 (32)
Alcoholic cirrhosis	24	3 (13)
Acute hepatitis	67	33 (49)
First-time blood donors & laboratory workers (males)	245	30 (12)
Hospital staff (screening programme)	569	23 (4)
Pregnant mothers	2273	101 (4)
Total	3087	154 (5)

Figures in parentheses depict percentage

Table 5 Prevalence of anti-HBc among liver disease patients and healthy subjects (Tested by RIA, ELISA)

	No. tested	No. positive
Primary liver cancer	18	17 (94)
Chronic hepatitis	40	37 (93)
First-time blood donors & laboratory workers (males)	52	14 (27)
Hospital staff (screening programme)	453	135 (30)

Figures in parentheses depict percentage

Table 6 HBV markers in primary liver cancer, cirrhosis and normal controls

	HBsAg	Anti-HBs	Anti-HBc	Any markers
Primary liver cancer	119/187 (63.6)	20/48 (41.7)	76/81 (93.8)	30/30 (100)
Non-alcoholic cirrhosis	28/87 (32.2)	3/8 (37.5)	58/69 (84.1)	–
Normal controls	23/569 (4.0)	171/564 (30.3)	182/568 (32.0)	207/564 (36.7)
Difference between HCC and normal controls	$p < .0001$	NS	$p < .0001$	$p < .0001$
Relative risk	41.5		32.2	∞

Figures in parentheses depict percentage

cer have a significantly higher frequency of HBsAg and anti-HBc than normal controls (Table 6). All liver cancer patients tested showed evidence of HBV infection (any HBV marker being positive) compared with 36.7 per cent of the normal population ($p < 0.0001$). On the other hand, the frequency of anti-HBs, the protective antibody against HBV, was not significantly elevated among liver cancer patients.

(i) Relationship between HBsAg and liver cancer among different ethnic groups

The age-standardized incidence of primary liver cancer is highest among Chinese followed by Malays and Indians; within each ethnic group, there is a male predominance. The HBsAg carrier frequencies of both males and females in these ethnic groups appear to parallel the liver cancer incidence of the respective ethnic group. Figure 2 shows the relationship between the prevalence of HBsAg and the incidence of primary liver cancer among the Chinese, Malay and Indian ethnic groups, divided into both males and females. There is a remarkable correlation between the HBsAg carrier and primary liver cancer rates among both males and females. For males, the equation is $Y = -1.53 + 2.29X$ ($r^2 = .99$); for females, $Y = 2.83 + .76X$ ($r^2 = .99$). The effect of HBsAg appears to be stronger in males than in females; for every unit increase in the HBsAg carrier rate, the corresponding increase in the incidence of primary liver cancer is 2.29 per 10^5 per year in males and only 0.76 per 10^5 per year in females. If HBV is one of the aetiological agents for primary liver cancer, a direct inference from this data is that if the carrier rate can be reduced (e.g. by vaccination) we may expect a drop in the incidence of liver cancer and this reduction can be expected to be more marked among males.

(ii) Relationship between types of HBV transmission and HBsAg carrier rates among different ethnic groups

Since there is such a remarkable correlation between HBsAg carrier rates and the incidence of primary liver cancer among different ethnic groups in Singapore, it may be

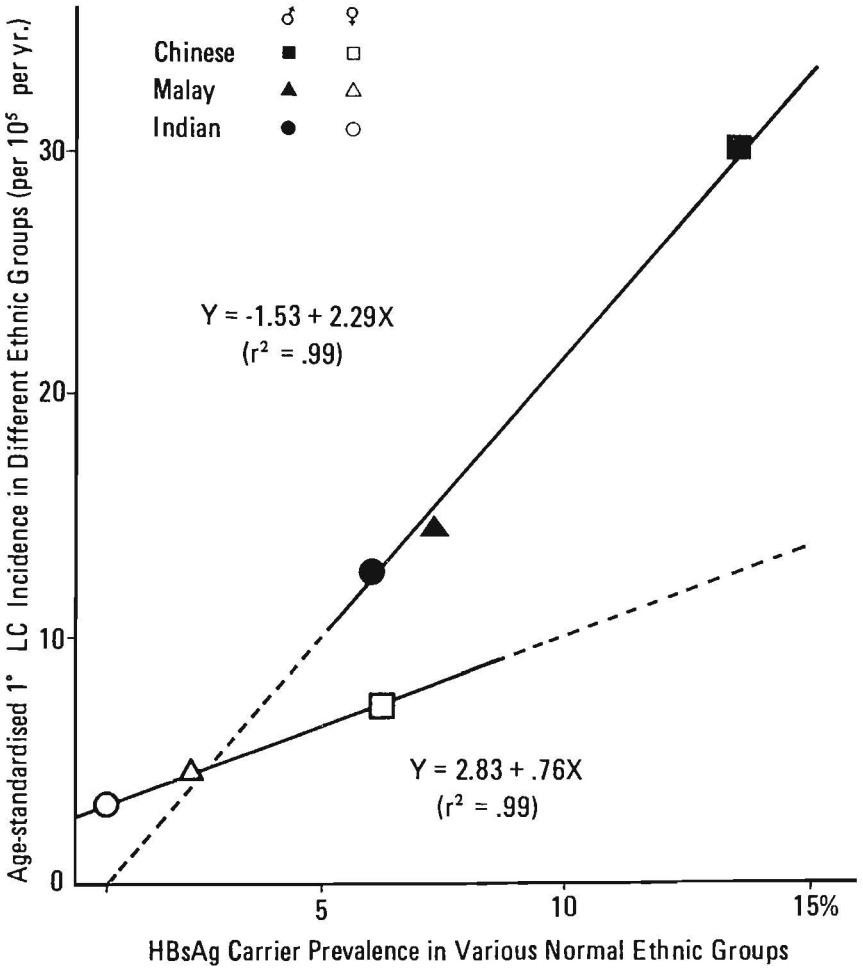

Fig. 2 Relationship between incidence of HBsAg and primary liver cancer among different ethnic groups. (LC = liver cancer)

Table 7 On-going projects on HBV in Singapore

Prospective study on carriers and development of liver diseases including HCC – Phoon, W.O.
Prospective studies on prevention of HBV infection, carrier status and liver diseases adults and children by the use of HBV vaccines – Oon, C.J.
Large scale production of HBV vaccine Production of reagents for HBV testing – Oon, C.J.

argued that the important type of HBV infection is the one that is most likely to generate carrier status; its ability to produce chronic carriers should reflect the differential carrier rates among the three ethnic groups. The generation of carrier status by perinatal transmission fulfills these criteria (Table 3). Furthermore, infection at such an early age is more likely to induce "permanent" carriers because of the relative immaturity of the immune system. On the other hand, it could be argued that HBV infection through acute hepatitis may be less important in the subsequent development of liver cancer because it occurs more frequently among Indians compared with Chinese; the lowest frequency of carriers is found among Indians. However, post-hepatitis carrier status has not been studied in our population and the carrier rate may be different among different ethnic groups following acute HBV hepatitis.

On-going projects

Table 7 summarizes some of our on-going projects related to HBV. With the ready availability of locally made HBV vaccine, projects will be concentrated on the effect of these vaccines in reducing chronic carriers, acute hepatitis due to HBV, chronic hepatitis and primary liver cancer. The ultimate proof that HBV causes primary liver cancer may be achieved through these intervention studies.

Conclusion and summary

HBV infection is endemic in Singapore. The majority of cases of infection are subclinical and infection occurs continuously among all age groups, about 50 per cent of the population being infected by 40-49 years of age and 100 per cent of the population in the > 60-year age group. The frequency of HBsAg is highest in the < 12-year age group. HBV is responsible for about 50 per cent of the acute viral hepatitis cases in Singapore.

Besides horizontal transmission, subclinical HBV infection also occurs vertically and perinatally. Maternal-infant transmission is most likely to occur among HBeAg-positive mothers and the majority of transmission occurs during the perinatal period. Perinatal transmission contributed about a quarter of the HBsAg carrier rate among one-year-old children.

HBsAg carrier status is not only strongly associated with primary

liver cancer but also in premalignant conditions of chronic hepatitis and non-alcoholic cirrhosis. There is a direct relationship between carrier status and primary liver cancer incidence in both males and females of the three ethnic groups: Chinese, Malays and Indians. There is some indirect evidence to suggest that HBV infection by perinatal transmission may be more important than infection by acute clinical infection in subsequent development of primary liver cancer.

REFERENCES

1. Yap EH, Ong YW, Simons MJ, Okochi K, Mayumi M, Nishioka K. Australian antigen in Singapore. II. Differential frequency in Chinese, Malays and Indians. Vox Sang 1972; 22:371-5.
2. Ong YW. Some clinical and epidemiological aspects of HBs antigenaemia and alpha-fetoprotein levels in Singapore. In: Liver Cancer, cancer problems in Asian Countries. Proceedings of the AFOCC, 2nd Asian Cancer Conference, 1976:57-60.
3. Quak SH, Singh R, Oon CJ, Wong HB. The immune status of Singapore children to hepatitis B virus. Aust Paediat J 1983; 19: 100-3.
4. Chan SH, Tan KL, Goh KT, et al. Maternal-child hepatitis B virus transmission in Singapore. Intr J Epidemiology 1984; in press.
5. Chan SH, Oon CJ, Seah CS. Acute viral hepatitis in Singapore. Lancet 1981; 2:469.
6. Committee on Epidemic Disease, Ministry of Environment, Singapore. Epidemiological News Bulletin 1979; 5:50-4.

Experimental and Clinical Results with the *Corynebacterium granulosum*-derived Immunomodulator P40*

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The demonstration that suspensions of heat-killed whole cells of anaerobic corynebacteria are capable of stimulating the natural defenses of an organism has aroused great interest during the two last decades. However, it is possible that the complexity of the intact bacterial cell might obscure the interpretation of experimental data. Furthermore, the high probability that administration of whole bacterial cells to man would elicit untoward reactions could limit their use in human therapy. As a result, many recent investigations have been aimed at isolating fractions from whole bacterial cells which would exhibit either all or only some of the immunostimulating activities of the intact cell. Most work in this field has been concentrated on attempts to obtain water-soluble fractions, as well as fractions which would be possible to synthesise chemically. Considerable achievements have already been attained. In general, myçobacteria have been used for fractionation, while only a few fractionation studies have involved anaerobic corynebacteria. With anaerobic corynebacteria, fractionation has been carried out exclusively on *C. parvum*. For our own investigations, we have used *C. granulosum* for the following reasons.

In the genus "anaerobic *Corynebacterium*" a strain belonging to one particular species may be active, whereas another strain of the same species may be inactive. In addition, an active strain may lose spontaneously its original reticulostimulating activity. A systematic study over a period of several years regarding the immunostimulating capacity of strains belonging to various species of anaerobic corynebacteria showed us[1] that a strain of *C. granulosum* (strain No. 5196) had preserved its reticulostimulating activity, whereas strains of *C. anaerobium* and *C. parvum* had lost practically all of their initial activity. Therefore, strain No. 5196 of *C. granulosum* was chosen for our studies.

This strain of *C. granulosum* was found to stimulate strongly the reticuloendothelial system (RES),[1] to protect mice against infection with *E. coli* 111 B4 and to accelerate the clearance of *E. coli* 111 B4 from the blood stream,[2] and to increase significantly the phagocytic activity of rat circulating leukocytes the phagocytic activity of which had been strongly impaired by extensive thermal injury of the skin surface of the rats.[3] The *C. granulosum* stimulation also resulted in a high increase of the complement activity of sera from thermally injured rats.[4] *C. granulosum* can also substitute for mycobacteria in Freund's complete adjuvant to induce a delayed-type hypersensitivity (DTH) reaction to the simple chemical: azobenzene-arsonate-N-acetyl-tyrosine (ABA-TYR).[5] The production of antibodies directed to sheep red blood cells (SRBC) was also strongly enhanced.[5] Furthermore, *C. granulosum* was found to inhibit the growth of various experimental grafted tumours: Ehrlich tumour,[2] mouse L1210 leukaemia and Friend leukaemia.[5]

Cell walls were isolated from whole cells of *C. granulosum* by using various treatments which resulted in either partly purified cell walls or purified cell walls.[5,6] The cell wall preparations thus obtained were found to exhibit biological activities close to those of the whole cells but, on a weight basis, cell wall preparations were not more active than whole cells. It is also interesting to note that the injection of *C. granulosum* cell walls and SRBCs resulted in two peaks of antibody response to SRBCs, one

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on day 14 and the other on day 28.[5] Thus, it appeared to us that active fractions could be isolated from anaerobic corynebacteria.

Migliore-Samour *et al*[7] reported on the isolation of a water-soluble fraction from *C. parvum* which exhibited strong adjuvant activity. These investigators fractionated whole cells of *C. parvum* by a technique which they had successfully used to fractionate mycobacterial cells. However, when we applied their technique for the fractionation of *C. granulosum* whole cells, we recovered the immunostimulating activities in a particulate fraction which we designated as P40 fraction. Here we report on the preparation of this fraction, its partial chemical characterisation and its main biological activities. We also include the initial results which have been obtained with the P40 fraction in clinical trials. Characteristics of P40 are represented in Table 1.

Production of large quantities of *C. granulosum* whole cells

C. granulosum bacteria were cultivated in fermentors in volumes of 300 or 700 litres. The medium consisted of liver-meat broth supplemented with 4 g of yeast-extract (DIFCO), 2.5 g of NaCl and 10 g of glucose per litre of medium. The pH was adjusted to 7.2-7.3. Cultures were incubated in a nitrogen atmosphere at 37°C and bacteria were harvested in a Sharples centrifuge (Type MV 12-RV 5 P1) after 48-72 hours. The bacteria were washed three times with saline and resuspended in saline before being heated in a water bath at 60°C for one hour. The biological activity of various batches was found to be quite comparable.[8]

Preparation of P40 fraction

Fractionation of *C. granulosum* bacteria was carried out on the delipidated cells as represented schematically in Figure 1. Delipidation was performed according to the method of Aebi *et al.*[9] The delipidated bacteria were disintegrated mechanically with the aid of an Omni-Mixer and were centrifuged at low speed to remove intact bacteria and large bacterial debris. The supernatant was subsequently fractionated with $(NH_4)_2SO_4$ and the fraction precipitated at 40 per cent saturation was harvested and dialysed exhaustively with distilled water. The dialysate was lyophilised. This fraction was designated as P40 fraction. The P40 fraction batches which resulted from the processing of different lots of *C. granulosum* whole cells exhibited comparable biological activities, indicating that the procedure was reproducible.

As calculated from the dry weight of delipidated bacterial cells, the P40 fraction was recovered with a yield in the range of 30 per cent.[8]

Main physico-chemical characteristics of P40 fraction

P40 fraction is insoluble. Examination under the electron microscope shows that its structure resembles pieces of bacterial cell wall (Fig. 2). All attempts to degrade it into a soluble derivative were unsucessful. These attempts included

Table 1 Immunomodulator P40:

1. Accelerates	CLEARANCE of carbon particles and pathogenic agents
2. Increases	RESISTANCE to infections
3. Enhances	ANTIBODY production
4. Restores	IMMUNE CAPACITY
5. Induces	DELAYED HYPERSENSITIVITY
6. Inhibits or delays	TUMOUR GROWTH

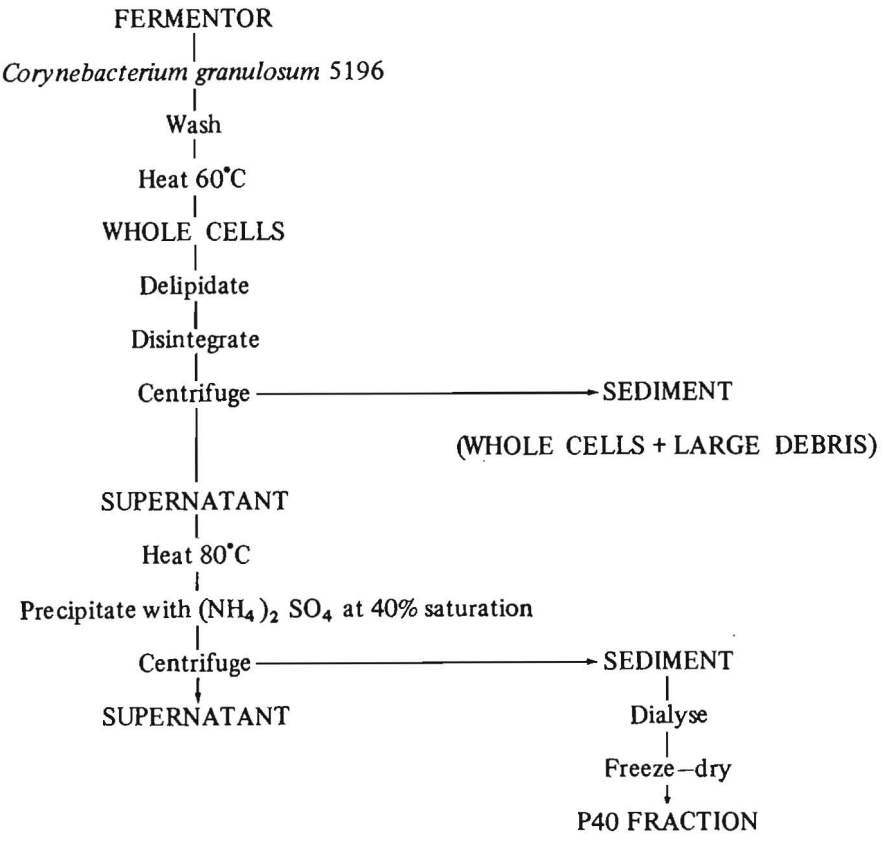


Fig. 1 Preparation of P40 fraction

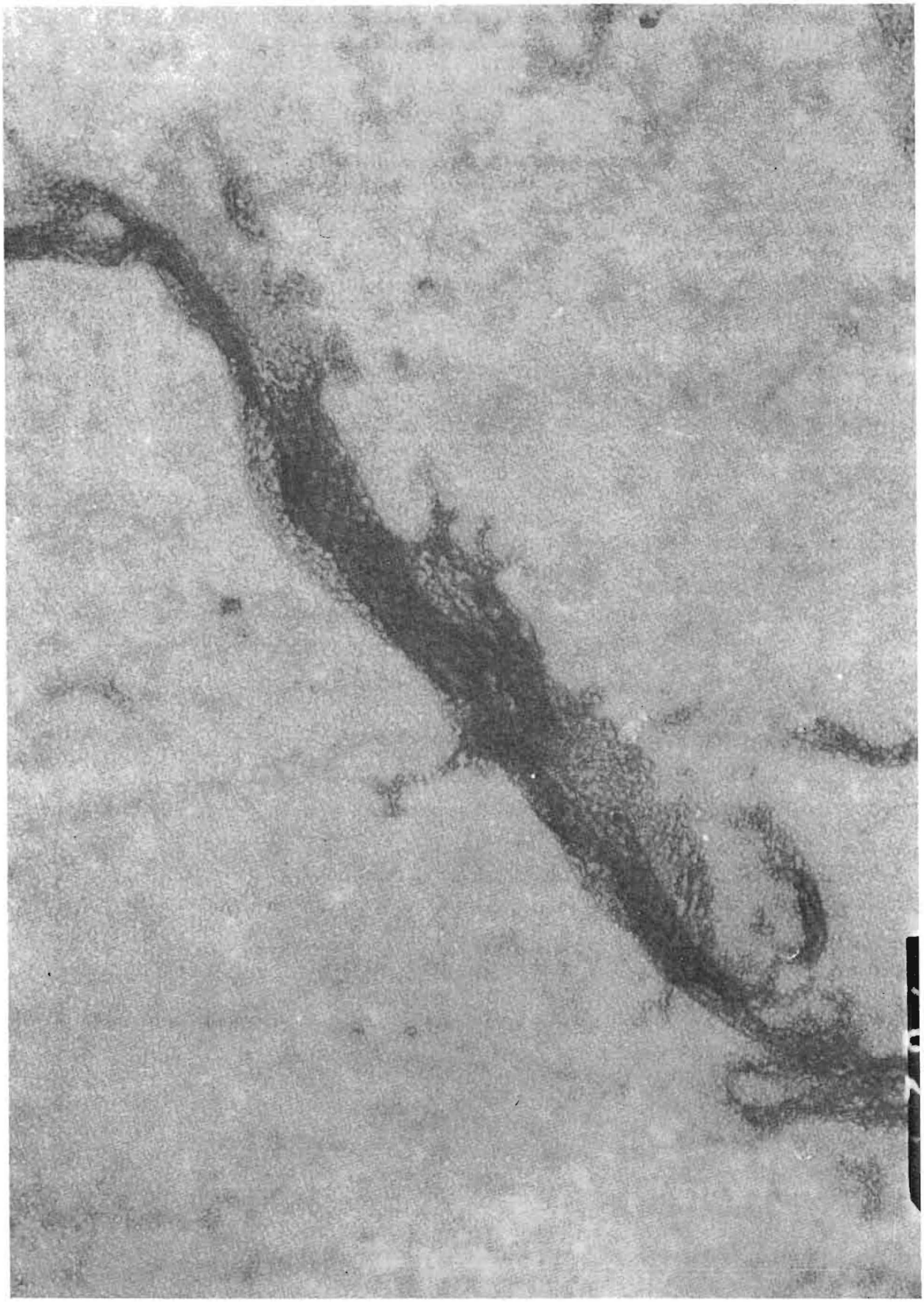


Fig. 2 Electron microscopic appearance of P40 fraction (magnification x 60,000)

enzymatic degradation with pronase, lysozyme, trypsin, chymotrypsin, RNase and DNase used either separately or sequentially. Chemical reagents such as 0.1 NaOH, 9% HCOOH, or 1% SDS or 6M guanidine in the presence or in the absence of 1% 2-mercaptoethanol were also ineffective in degrading P40 fraction. Therefore, it appears that the P40 fraction has a rigid, compact structure within which the bonds sensitive to breakdown by enzymes and chemical reagents are inaccessible. This also explains why a water suspension of the P40 fraction can be autoclaved at 120°C for 20 minutes without impairment of its immunostimulating activity. Consequently, suspensions of the P40 fraction can be sterilised by autoclaving, which eliminates the necessity to add formaldehyde which is necessary in whole-cell suspensions.[8]

Table 2 Chemical composition of P40 fraction

Amino acids	g/100 g P40 (Dry weight)	
His	1.69	
Lys	3.11	
Arg	3.09	
Asp	3.71	
Thr	1.97	
Ser	1.37	
Glu	6.34	
Pro	1.09	
Ala	3.38	
Gly	3.31	
Cys	0.30	
Val	2.88	
Met	1.10	
Ile	1.79	
Leu	3.04	
Tyr	0.91	
Phe	2.15	
Try	1.07	
Total		42.30
Dap		3.23
Neutral sugars		13.67
Hexosamines		10.09
Muramic acid		10.38
Ash		0.83
Total		80.40

As summarised in Table 2, P40 fraction is composed of all the amino acids which are usually present in proteins, as well as diaminopelemic acid, muramic acid, and neutral and amino sugars. On a weight basis, the composition accounts for about 80 per cent of the P40 fraction. The missing 20 per cent could have been lost by destruction of the amino acids or sugar residues, or both during acid hydrolysis of the fraction since the simultaneous presence of amino acids and sugar residues can result in losses of either one by formation of complexes during glycoprotein hydrolysis.

Table 2 also shows that glutamic acid, glycine, aspartic acid, and alanine are the major amino acids. Large amounts of neutral sugars and hexosamines were also present. The neutral sugars identified were glucose, galactose, mannose and ribose and the hexosamines were glucosamine and galactosamine, which are present in a molar ratio of 7 to 2. The presence of both diaminopimelic acid and muramic acid indicates that the P40 fraction is derived, at least in part, from the bacterial cell wall.[8]

It is hard to provide a clear interpretation of the analytical results. Because the P40 fraction is insoluble, standard procedures cannot be used to try to resolve its possible complex nature. Therefore, we are not yet in a position to decide whether the P40 fraction comprises only one molecular entity or a mosaic of separate molecular entities, which would be associated in a highly stable structure. However, the presence of diaminopelemic acid and muramic acid is consistent with the existence of a peptidoglycan-like structure in P40. To confirm this assumption, it would have to be shown that diaminopelemic acid is present as its L, L-isomer. In the light of our present knowledge, we may speculate that the P40 fraction is likely to consist of the association of the cell-peptidoglycan and a glycoprotein.

Biological activities of the P40 fraction

a) Stimulation of the reticuloendothelial system:

Like *C. granulosum* whole cells, the P40 fraction was found to be a potent activator of RES. It is capable of increasing both granulopexic capacity (carbon clearance test) and phagocytic activity (enhanced resistance to infections with virulent bacteria). The granulopexic activity of P40 as expressed by the K/K_O index (namely, by the ratio of the activity measured in stimulated mice to that measured in control non-stimulated mice) attained its maximum value about 6-7 days after stimulation with the P40 fraction. A mean value of 4 was found for the K/K_O index of various P40 batches. The increase in granulopexic activity is not correlated with the increase in weight of both spleen and liver; in fact, the spleen and liver continued to grow after the granulopexic capacity had returned to normal values.[2,8]

The P40 fraction is also capable of enhancing the phagocytic and bactericidal activities of macrophages as is evidenced by increased resistance of mice to infection with weakly virulent bacteria (*E. coli* 111 B4, P. Lallouette *et al*)[2] and with strongly virulent bacteria such as *L. monocytogenes* that develops intracellularly (Fig. 3). The increase of the bactericidal activity of macrophages from mice stimulated with P40 is also correlated with the potentiation of their enzymatic activities (K. Masek: personal communication).

The preventive effect of P40 on the experimental *E. coli* infection of the upper and lower urinary tract was investigated in rats.[10] Two-thirds of the rats infected by the lower route were protected against the infection. Under our

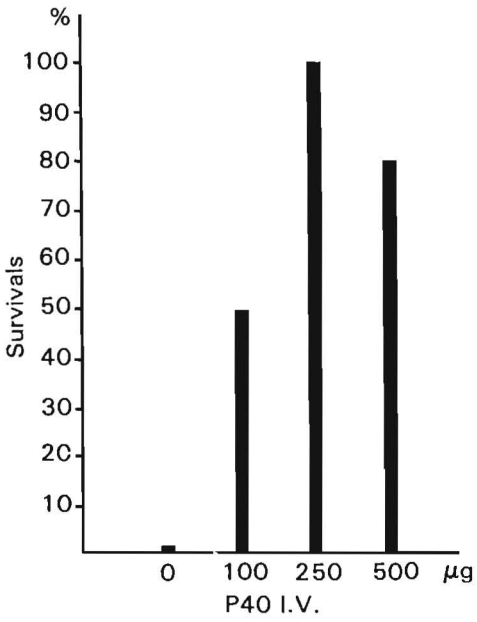


Fig. 3 L. monocytogenes (1,6 LD_{50})

conditions, the bacteriological picture of the infection of rats, which were infected by the upper route, was only slightly influenced but the development of nephropathia was significantly decreased.

b) Effect on antibody production:

The ability of P40 to enhance the production of antibodies in mice in response to an i.v. injection of sheep red blood cells (SRBC) in saline showed that the adjuvant effect of P40 was maximum at 250 µg per mouse when injected seven days before the SRBC stimulus. Furthermore, the antibody level was at its peak about 6-7 days after the SRBC stimulus was given. For the experiments, a dose of SRBC was chosen that elicited only a negligible antibody response in control, unstimulated mice. Under these conditions the haemagglutinin content remained at a definitely low level in the sera of those mice which were not stimulated with the P40 fraction. In contrast, sera of those mice which were stimulated with the P40 fraction contained high levels of haemagglutinins. The antibody content of these sera decreased slowly; compared with the sera of mice not stimulated with the P40 fraction, antibody content was 11 times higher four weeks later, and three times higher 16 weeks later in the sera of mice stimulated with the P40 fraction.

The effect of the P40 fraction was also investigated with regard to a secondary response of mice boostered 16 weeks after their priming with SRBC. The secondary response in mice which had been stimulated with the P40 fraction seven days before the primary SRBC immunisation was found to be about 4 times higher than that in mice which had not received the P40 injection. Moreover, when the P40 injection was given seven days before giving the mice the SRBC booster, the secondary response was enhanced about 50 times.[8] Other investigators reported an increased production of haemagglutinins in mice on day 5 after a single i.v. injection of 250 µg of P40 given seven days before the i.v. immunisation with SRBC in dose varying from 10^5 to 10^9 SRBC per mouse. Increased production of antibodies was verified for all doses of SRBC injected, except for the dose of 10^5 cells. The major adjuvant activity of P40 concerned the production of IgM- or 2-MER-sensitive antibodies.[11]

Experiments were also performed using tetanus toxoid as a soluble antigen. In this instance, the enhancing effect of P40 on the formation of antibodies was assessed by measuring the amount of toxoid corresponding to one 50 per cent protective dose (1 PD_{50}) comparatively in mice stimulated with the P40 fraction and in mice not stimulated. In P40-treated mice, the amount of toxoid corresponding to 1 PD_{50} was found to be decreased with respect to that of unstimulated mice (unpublished results).

c) Immunorestorative effect:

It was also verified that the P40 fraction could counteract the immunosuppressive effect of the cyclophosphamide, as measured by the production of haemagglutinins in mice.[12] In fact, the P40 fraction is capable of abolishing not only the immunosuppressive action of cyclophosphamide, but also of enhancing the antibody response of cyclophosphamide-suppressed animals to a primary immunisation with SRBC. However, to counteract the action of cyclophosphamide, the P40 fraction has to be administered before the immunosuppressive drug. Furthermore, cyclophosphamide must not be used at doses which would completely suppress the immune responsiveness of the mice.

These results have been extended by S. Ben-Efraim (Tel Aviv University) who verified in BALB/c mice that the immunosuppressive effect of cyclophosphamide and of methotrexate on contact hypersensitivity to DNFB could be counteracted by the i.v. administration of P40 prior to the injection of the immunosuppressive drug.

A DTH reaction to either ovalbumin or ABA-Tyr was induced when the antigen or the hapten was injected in emulsion in Freund's complete adjuvant in which mycobacteria were replaced by P40 fraction (unpublished results). Recently, an exhaustive study [12,13] of the capacity of P40 fraction injected in saline showed an immunopotentiation of DTH in mice immunised with supraoptimal doses of antigen which would normally depress DTH in control mice. In contrast, when the P40-stimulated mice were immunised with optimal or suboptimal doses of SRBC for induction of DTH in control mice, lower DTH levels were also observed in P40-stimulated mice. This immunoregulatory effect of P40 was ascribed to the accelerated catabolism of the antigen and to the T-cell adjuvant effect of the P40. A significant enhancement of DTH was also recorded when the SRBC were injected s.c. together with the P40 fraction. The strongest effect was observed for a dose of 5 µg of P40 injected along with 10^8 SRBC. It was further established that the observed

potentiation of DTH did not rely only on a depot phenomenon, as is seen with other adjuvants. Furthermore, the adoptive transfer of DTH indicated that the P40-induced enhancement of DTH could be ascribed to the adjuvant effect of P40 on the lymphoid cells, and not to a non-specific action on accessory cells.

In parallel experiments, the capacity of P40 to induce a state of DTH to certain of its antigens was demonstrated using four different immunological parameters as correlates of cellular immune response.[13] The results obtained suggest that P40 systemic hypersensitivity might be of the first type as opposed to the tuberculin response that is of the second type.

Anti-tumour effect of the P40 fraction

The anti-tumour effect of P40 fraction has been investigated using several experimental tumour models.

When mice were stimulated by a single i.p. injection of 10 μg of P40, seven days before the i.p. grafting of 10^5 Ehrlich tumour cells to mice, inhibition of the tumour growth was achieved in 100% of the animals.[2]

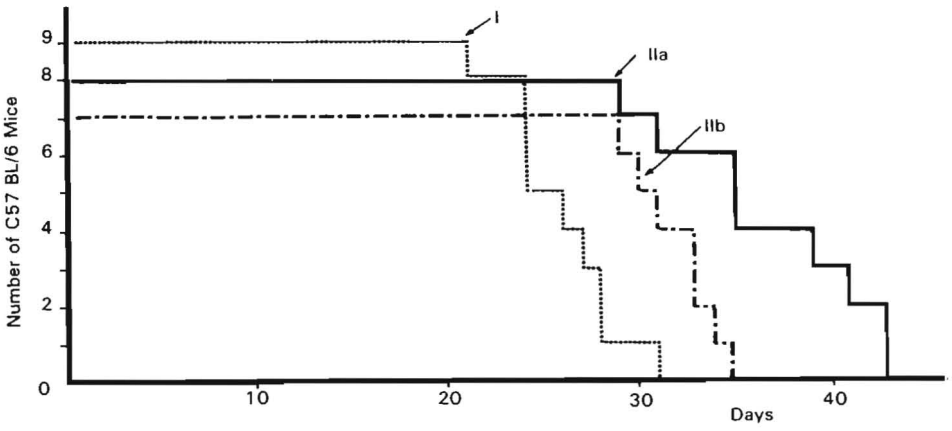
With P815 mastocytoma, the grafting of the tumour in its ascitic form was carried out both in non-isogenic MLTB mice and in isogenic DBA/2 mice. This type of tumour was successfully transplated in MLTB mice by Lalouette and Schwartz and has been serially transplanted in this mouse strain for several years. Although the tumour has lost much of its virulence by transplantation from DBA/2 mice to MLTB mice, an injection of 10^5 cells will kill all recipients in about 28 days. Spontaneous regressions of established tumours have never been observed. The P40 fraction was injected i.p. at a dose of 100 μg per mouse at various times from one day to 9 nine days after the grafting of P815 mastocytoma to MLTB mice. Under these conditions, 100 per cent tumour rejection was obtained when the P40 fraction was injected up to six days after the tumour grafting. Injected nine days after the tumour challenge, the P40 fraction exercised no effect on tumour growth. The ineffectiveness of the P40 fraction when administered nine days after grafting the tumour is probably due to the fact that the population of tumour cells is too large at this time to be overcome by the immune defenses of the recipient. It was also observed that the P40 fraction was effective only when given i.p. and not when given i.v. In similar experiments in which *C. granulosum* whole cells were used in place of the P40 fraction, tumour growth was only retarded.

When the P815 mastocytoma was grafted to syngeneic DBA/2 mice, the P40 fraction did not influence tumour growth. However, protection could be afforded to a certain extent when the P40 fraction was injected before grafting the tumour.[2]

The Lewis lung carcinoma was investigated as an alternative tumour. It was maintained in its solid form by serial subcutaneous injection in C57B1/6 mice. In the experiments with Lewis carcinoma, the effect of a single i.v. injection of the P40 fraction at a dose of 250 μg per mouse was first studied. This was given either seven or three days before an i.m. challenge with $2x10^4$ tumour-cells, or given on the same day, or three, six or nine days after the tumour challenge. The results showed that the P40 fraction given before or at the same time that the tumour was grafted accelerated the tumour growth as estimated by measuring the variation of the diameter of the leg injected with the adjuvant as a function of time. However, no statistically significant difference was noted with regard to survival time when compared with control animals. However, when the P40 fraction was injected three days or six days after the tumour challenge, the tumour growth was significantly retarded. Given nine days later, the P40 fraction was without effect on tumour development.

Subsequently, we determined the optimum dose of the P40 fraction when injected i.v. four days after the tumour challenge. Under these conditions, the inhibitory effect of P40 on tumour growth was maximum at a dose of 100 μg. At this dose, the prolongation of the survival of the mice was highly significant. At doses of 250, 500 and 1,000 μg, the effect was less marked but still significant (Fig. 4).[8]

Castro Faria and Grynberg[14]



I : Control mice
II : Treated mice : one I.V. injection of a) 100 μg P40 and b) 500 μg P40 4 days after the tumour graft

Fig. 4 Lewis tumour

have used glycerol-induced vesicles from tumour-cell membranes as a specific tumour antigen. The association of this antigen with P40 after a single i.p. injection resulted in 70-80 per cent-complete regression of a syngeneic methylcholanthrene-induced fibrosarcoma. P40 alone or the vesicles alone were ineffective.

Using L1210 leukaemia as a tumour model in syngeneic (C57B1/6xDBA/2) F1 mice, the effect of P40 alone on the tumour was compared to that of its association with other treatments.[15-19] The i.p. injection of a single dose of 100 μg of P40 seven days before the grafting of $1x10^5$ L1210 tumour-cells resulted in a significant prolongation of the survival time of the P40-treated mice as compared with the control mice. When glutaraldehyde-inactivated tumour cells (GA-L1210) or glutaraldehyde-inactivated L1210 tumour cells to which tetanus toxoid was coupled (GA-L1210-Tet) were injected seven days before the tumour challenge, tumour growth was not retarded and even facilitated. When two injections were given seven days apart and seven days before the tumour challenge, similar results were recorded. In contrast, the administration of inactivated cells with P40 under the same conditions as above resulted in a highly significant prolongation of the survival time (more marked with the combination of GA-L1210-Tet with P40) as compared with that of the control animals.

In subsequent studies,[17,18] the effect on L1210 leukaemia of chemotherapy alone, with either daunorubicin or mitomycin, was compared with immunotherapy with P40 alone as was the association of P40 and GA-L1210-Tet with chemotherapy. The treatment consisted of four injections on days -14 and -7 and on days +4 and +11. Chemotherapy alone and P40 treatment alone afforded the same degree of protection. In contrast, the combination of chemotherapy with P40 and GA-L1210 and P40 resulted in a significant increase of survival time in comparison with chemotherapy alone or P40-treatment alone. The survival of a few animals was even recorded under specified conditions. Furthermore, the rechallenge of surviving mice with the same number of L1210 tumour cells showed that about 50 per cent of the animals had developed specific resistance to the tumour.[17,19]

It appears from these results that the combination of chemotherapy with active immunisation and non-specific immunostimulation is more efficacious than single treatments for the control of L1210 leukaemia. Consequently, we are currently carrying out additional experiments in order to specify more precisely optimal conditions for combined treatments.

Experimental DMBA-induced mammary carcinoma was also used in female Sprague Dawley rats as described by Huggins *et al.*[20] The animals fed with DMBA developed mammary tumours beginning the seventh week after administration of the drug. P40 was given either intratumourally (i.t.), or peritumourally (p.t.), or intravenously (i.v.). Two weeks after P40-treatment, all rats underwent surgery for the removal of tumours. A second group of rats received P40-treatment at the time of appearance of the first tumour having an estimated volume greater than 0.6 $cm.^3$ P40 was then injected i.t.

In the first group, no significant difference in the mean survival time was recorded with respect to control rats not receiving P40. In contrast, the volume of tumoural recurrence was highly significantly decreased in the P40-treated rats. Very similar results were obtained in the second group.

Toxicology

A thorough toxicological examination of the P40 fraction has been carried out in rats and dogs. These experiments showed that the P40 fraction was devoid of any acute and chronic toxicity at doses far beyond the therapeutic doses. The absence of undesirable effects was subsequently confirmed in clinical trials.

Clinical trials with the P40 fraction

Since the P40 fraction was devoid of toxic effects at a dosage up to 50 times that intended to be used in humans, clinical trials were initiated in patients presenting either of two kinds of pathological conditions: recurring infections and neoplasia.

Treatment of recurring infections:

It appears very likely that recurrences of chronic infections may originate from the patient's inability to mount an adequate cell-mediated reaction against the infecting agent. Therefore, it appears to us that stimulation of the patient's natural defenses might substitute for specific immunisation.

To test this hypothesis, 117 patients suffering from recurring infections of the respiratory tract were divided into three groups for treatment (Table 3): Group I received either mono- or poly-microbial preparations (vaccinotherapy); Group II received vaccinotherapy combined with P40, and Group III received P40 alone. Comparison of the effectiveness of the various treatments showed that patients in Groups II and III were significantly improved (on the basis of three criteria) in comparison to Group I patients ($p<0.001$).[21,22]

Subsequently, a trial performed in randomly selected patients with recurring respiratory infections (mostly chronic obstructive bronchitis) confirmed that P40 treatment results in the suppression of symptoms, in a decrease in the number of infectious episodes, and in a decrease in the number of cumulative days of antibiotherapy for the year during which P40 treatment was administered as compared with the year prior to the initiation of P40 treatment.[24]

P40 treatment was also given to patients with recurring herpetic cutaneo-mucous infections. Of 12

Table 3 Comparative statistical evaluation of the effectiveness of vaccinotherapy alone (group I), vaccinotherapy + P40 (group II) and P40 alone (Group III) in the treatment of patients with relapsing chronic infections of the respiratory tract.

Treatment	No. of patients	Proportion of improvement according to criteria	% of patients improved
Group I	79	18/79	22.78
Group II	21	18/21	85.71
Group III	17	14/17	82.35

Group I – II :		$p < 0.001$	(highly significant)
Group I – III :		$p < 0.001$	(highly significant)
Group II – III :	$0.9 <$	$p > 0.5$	(not significant)

patients who received one or a few injections of P40 0.20 mg/ml during the presence of a herpes blister, nine of them experienced a dramatic shortening of the duration of their blister which faded in 24-48 hours instead of 8-10 days as was the experience of patients in the control group. Of 24 patients who presented frequent recurrences of herpes, 19 did not have recurrent herpes over a period of time ranging from one to four years after 10 injections of P40 spaced one week apart.[21]

P40 treatment was also successful in treating patients with either vaginal candidiasis or *E. coli* cystitis. Patients improved by the treatment (about 75%) have not experienced relapses over a period of time ranging from one to four years post-treatment.[21,25]

We must emphasise that skin tests for delayed hypersensitivity with recall-antigens (bacteria from the respiratory tract, SK-SD, PPD, candidin, *E. coli*) which were negative before P40 treatment became positive after the treatment in improved patients. In particular, in a series of 10 patients with vaginal candidiasis, who were unresponsive to candidin, eight of them showed improvement and at the same time they became responsive to candidin. In contrast, the two patients who were not improved by the P40 treatment also remained unresponsive to candidin.

Table 4 Evaluation of the cutaneous response to recall-antigens before and after treatment with P40 fraction (n = 67)

Score	No. Responsive/total Before P40	After P40
4+	41/67 (61%)	63/67 (94%)
5+	31/67 (46%)	55/67 (82.%)
6+	27/67 (40%)	45/67 (67%)
7+	18/67 (27%)	32/67 (48%)

The importance of performing skin tests on patients with recurring infections as a guide for therapeutic decision-making and as a test to evaluate the efficacy of the treatment has previously been emphasised.[26] In this connection, the possibility of modulating skin response to an antigen by P40 fraction has been reported.[27] The P40 fraction would either enhance a poor skin reaction or weaken an exaggerated skin reaction as a function of the ratio of P40 to antigen.

P40 treatment in breast cancer patients

Skin-testing of breast cancer patients with recall-antigens showed that a large proportion of them were immunosuppressed, since their scores turned out to be much lower than those of a control population of individuals apparently free of cancer.[28,29] In fact, the exploration of the immune status of patients with breast cancer was performed using skin tests with five different recall-antigens to evaluate their DTH responses. Each patient was assigned a composite score based on the intensity of the skin test response to the five antigens (Table 4). The results showed that the frequency of anergy was significantly higher in breast cancer patients than in a control group of individuals apparently free of cancer. Furthermore, for patients with mean DTH scores, a significant difference was found between those who had lymph node involvement and those who had no lymph node involvement. In the former patients, fewer showed mean scores when compared with the latter. A significant difference as a function of age was found only for the highest scores.[20,30]

An attempt to restore the DTH reactivity of the immunosuppressed cancer patients by P40 treatment (1 subcutaneous injection of 0.2 mg three times weekly for three weeks) resulted in a significant decrease in the incidence of anergy. Lymph node involvement had no influence on the issue of P40 treatment. Thus, P40 treatment restored DTH reactivity in about half or more of the patients according to the type of antigen used for the skin tests (Tables 4 and 5).

Evaluation of the effect of P40 treatment on the survival rate of breast cancer patients is currently being carried out.

Summary

We report the preparation of an immunostimulating fraction from mechanically disrupted, delipidated cells of the anaerobic *Corynebacterium granulosum*. Since this fraction is precipitated by ammonium sulphate at 40 per cent saturation, it has been designated as P40 fraction. P40 is a particulate substance appearing in the electron microscope as pieces of the bacterial cell wall. The chemical composition of P40 is consistent with the assumption that it consists of an association of the cell wall peptidoglycan

Table 5 Comparison of the results obtained in breast cancer patients before and after treatment with P40 fraction and with controls when skin tested with recall-antigens. Results are expressed as per cent of positive reactions

Tests	Patients		Controls
	before P40	after P40	
PPD	61	83	74
Candidine	39	73	80
Divasta	42	67	76
Streptococci	34	61	85
Streptococcal exoproteins	18	40	74

together with a glycoprotein. P40 fraction can exercise a broad spectrum of activities on the immune system: it can 1) stimulate RES; 2) increase nonspecifically the resistance of animals to various bacterial infections; 3) enhance the production of antibodies to SRBC; 4) counteract the immunosuppressive effect of cyclophosphamide and methotrexate; 5) induce delayed-type hypersensitivity to various antigens and to itself; and 6) inhibit the growth of various experimental tumours.

Clinical trials have indicated that it is effective in the treatment of recurring infection of either the respiratory tract or the uro-genitary tract. In breast cancer patients, P40 was found to be capable of restoring cell-mediated immunity, as measured by skin tests with recall-antigens, in immunosuppressed patients.

REFERENCES

1. Raynaud M, Kouznetzova B, Bizzini B, Chermann JC. Etude de l'effect immunostimulant de diverses especes de Corynebacteries anaerobies et de leurs fractions. Ann Inst Pasteur 1972; 122:695-700.
2. Lallouette P, Bizzini B, Raynaud M. Etudes sur la phagocytose et sur des tumeurs greffees de la souris. Nouv Genie Med 1975; 25:13-20.
3. Jacques L, Mathieu D, Bizzini B. Variations du pouvoir phagocytaire des leucocytes circulants chez les rats brules. Path Biol 1978; 26:495-502.
4. Jacques L, Bizzini B, Mathieu D. Variation des activites complementaire et phagocytaire chez les rats brules. Effets d'une immunostimulation par *Corynebacterium granulosum*. Medecine et Mal Infect 1978; 8:512-8.
5. Kouznetzova B, Bizzini B, Chermann JC, Degrand F, Prevot AR, Raynaud M. Immunostimulating activity of whole cells, cell-walls and fractions of anaerobic corynebacteria. Recent Results in Cancer Res 1974; 47:275-93.
6. Prevot AR, Raynaud M, Bizzini B, Chermann JC, Kouznetzova B, Sinoussi F. Activite reticulostimulante des parois de Corynebacteries anaerobies. CR Acad Sci (Paris) 1972; 274:2256-8.
7. Migliore-Samour D, Korontzis M, Jolles P, Maral L, Floc'h F, Werner GH. Hydrosoluble immunopotentiating substances extracted from *Corynebacterium parvum*. Immunol Commun 1974; 3:593-603.
8. Bizzini B, Maro B, Lallouette P. Isolement et caracterisation d'une fraction dite fraction P40 a partir de *Corynebacterium granulosum*. Medecine et Mal Infect 1978; 8: 408-14.
9. Aebi A, Asselineau J, Lederer E. Sur les lipides de la souche humaine "Brevannes" de *Mycobacterium tuberculosis*. Bull Soc Chim Biol 1953; 35:661-84.
10. Mathieu D, Fries D, Auer J, Bizzini B, Jacques L. Action d'un immunomodulateur (P40) sur l'infection urinaire a *Escherichia coli* chez le rat. Biomedicine 1982; 36:420-5.
11. Fuster MJ, Lagrange PH. Immunomodulation with P40, an insoluble delipidated fraction of *Corynebacterium granulosum*. I. Immune response to sheep red blood cells. Ann Immunol (Inst Pasteur) 1982; 133 C:253-67.
12. Lallouette P, Maro B, Schwartz A, Bizzini B. Effet antagoniste d'une fraction isolee de *Corynebacterium granulosum* envers le pouvoir immunosuppresseur de la cyclophosphamide chez la souris. CR Acad Sci (Paris) 1976; 283:713-6.
13. Lagrange PH, Fuster MJ. Immunomodulation with P40, an insoluble delipidated fraction of *Corynebacterium granulosum*. II. Specific immune responses of the host. Ann Immunol (Inst Pasteur) 1982; 133C: 269-87.
14. Castro Faria H, Grynberg NF, Souza ASR, Gomes RM. Eradication of established murine tumor with plasma membrane vesicles and *Corynebacterium granulosum* fraction. Abstracts of the Fourth International Congress of Immunology, Paris, July 1980, N0. 10.5.11.
15. Relyveld EH, Bizzini B, Ben-Efraim S. Effet protecteur de la fraction P40 isolee de *C. granulosum* dans la leucemie L1210 de la souris. CR Acad Sci (Paris) 1981; 293:39-42.
16. Relyveld EH, Bizzini B, Ben-Efraim S. Prevention et traitement de la leucemie L1210 de la souris a l'aide de cellules tumorales inactivees et greffees, en association d'un immunomodulateur, la fraction P40, et d'agents antitumoraux. CR Acad Sci (Paris) 1982; 294:571-6.
17. Relyveld EH, Ben-Efraim S, Bizzini B. Immunoprophylaxis and immunotherapy of L1210 mouse leukemia using the immunomodulator P40 and inactivated and grafted tumor cells together with chemotherapy or not. Proc 13th Intern Cancer Congr Sept 8-15, 1982 Seattle (USA) 1982: 475.
18. Relyveld EH, Bizzini B, Ben-Efraim S. Prevention and treatment of L1210 leukemia using xenogenized tumor cells and non specific immunostimulation with P40 (fraction of *C. granulosum*) in conjunction with chemotherapy. Rev Inst Pasteur (Lyon) 1983; 16:140-1.
19. Relyveld EH, Bizzini B, Ben-Efraim S. Prevention and treatment of L1210 mouse leukemia by immunization with xenogenized tumor cells combined with immunostimulation by the P40 fraction of *C. granulosum* and chemotherapy. Int J Immunopharmac 1984; in press.
20. Reynier J, Villet R, Bazin JC et al. Immnological investigation and immunotherapy in patients operated on for breast carcinoma. Int Surg 1982; 67:17-24.
21. Henocq E, Bizzini B. P40 a biological immunomodulator in the treatment of human recurrent infections. Proc of the "Recent Advances on Immunomodulators", International Symposium, May 14-16, 1982 Viareggio (Italy).
22. Bizzini B, Henocq E, Reynier J, Ickovic MR, Ben-Efraim S, Relyveld EH. Preparation and characterization of the new immunomodulator P40, isolated from *C. granulosum*. Preliminary results obtained in the treatment of patients with relapsing chronic infections and with breast cancer. Proc 13th Intern Cancer Congr Sept 8-15, 1982 Seattle (USA) 1982:475.

23. Henocq E, Bizzini B, Bazin JC. Immunoadjuvants, vaccins microbiens et recidives des asthmes infectes. 10th Eur Congr Allergology Clin Immunol Sept 4-9, 1977 Prague (Czechoslovakia). In: Allergologia et Immunopathologia 1977;5:408.
24. Ickovic MR, Henocq E, Relyveld EH, Bizzini B. Effect of immunostimulation with the *Corynebacterium granulosum* derived immunomodulator P40 on patients with recurring respiratory infections. J Asthma 1984;21:29-33.
25. Henocq E, Bizzini B, Arvis G. Traitement des cystites recidivantes par un immunomodulateur biologique (P40). La Nouv Presse Med 1984: submitted for publication.
26. Henocq E, Bazin JC, Bizzini B. Exploration cutanee de l'immunite a mediation cellulaire dans les infections recidivantes. Consequences therapeutiques. Sem Hop Paris 1977;53:2271-2.
27. Henocq E, Bazin JC, Bizzini B, Reynier J. Adjuvant P40 et reactions cutanees a l'antigene. Medecine et Mal Infect 1978; 8: 415-21.
28. Santus JP, Henocq E, Bizzini B, et al. Cancer du sein operable. Appreciation dynamique de l'immunite cellulaire et de l'immunostimulation par l'adjuvant P40. Path Biol 1979;27:395-401.
29. Bizzini B, Henocq E, Bazin JC. Status immunitaire et immunostimulation. Rev Franc Endocrinol Clin 1978; 19:433-8.
30. Reynier J, Bazin JC, Bizzini B, et al. Cancer du sein. Comportement immunitaire. Immunostimulation par la fraction P40. Chirurgie 1980;106:48-54.

Unido's ICGEB: A Case of Petty Politics Prevailing Over Scientific Peer-Reviews*

Pornchai Matangkasombut, M.D., Ph.D.

The international scientific community should be informed of the development during the process of establishing the so-called ICGEB (International Centre for Genetic Engineering and Biotechnology) initiated by UNIDO (the United Nations Industrial Development Organisation) and gain from using it as a case study of how political manipulation can interfere with and prevail over the process of scientific peer-review. Biotechnologists and immunologists in developing countries in particular, whose work and opportunities can be directly or indirectly effected by this mishap should heed the tragic changes of events ensued.

Biotechnology including cell immortalisation through cell fusion and/or transformation and recombinant DNA technology has been well recognised as promising bases for a new era of scientific and technological revolution. With these tools, appropriate microbial, plant and animal cells can be manipulated according to design to enable production of higher quantity and quality of food stuffs, other agricultural and agro-industrial products, pharmaceuticals, biologically active macromolecules etc. In immunology itself these tools have already led to the elucidation of the molecular genetic bases of biosynthesis of specific antibodies; of cell interactions in immuno-regulation. The resultant libraries of cloned genes, of B-cell hybridomas and monoclonal antibodies; of cloned T-cell lines and cell interaction macromolecules including lymphokines, interleukins and one of the interferons; of cell surface differentiation, allotypic, idiotypic and anti-idiotypic markers have opened up the possibilities of elucidating detailed immunopathophysiological sequence of events at the cellular and molecular levels in a wide range of human diseases. These also make it increasingly possible the production of improved diagnostic reagents; and immunoprophylactic, immunotherapeutic and immunomanipulating agents at lower costs.

A wide gap of know-how already exists between the developed and the developing countries and with the rapid nature of development in these areas the gap can easily widen even more rapidly and promises to place the least developed countries at further disadvantage and an ever more technologically and economically dependent position.

A group of scientists mindful of international development recommended to UNIDO in 1981 that an international centre should be established to help developing countries to close this gap. In order for the centre to be successful and viable they also recommended a set of criteria based on scientific capability and socio-economic infrastructure for the selection of the location in a suitable host country. A meeting of interested countries at various levels of development was convened in Belgrade in December of 1982 in which participating countries enthusiastically endorsed the concept. As far as the location was concerned, since several countries proposed to host the centre, a decision could not be made and the meeting resolved that it should be in a developing country if there is one that meet the stringent set of scientific and financial criteria agreed upon and that a *Selected Committee* consisting of scientific experts appointed at the meeting be charged with the responsibilities of evaluating the relative suitabilities of each candidate host country on the basis of the scientific and socio-economic criteria above and that the results of evaluation of this committee will be the basis of a decision on the location to be made by a ministerial level meeting to be convened subsequently.

The *Selected Committee of Experts* proceeded to make extensive and intensive site visit in each of the candidate countries, i.e., Belgium, Cuba, India, Italy, Pakistan and Thailand spending 7-10 days in each country from March to May of 1983. They unanimously recom-

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mended that Thailand is the only developing country that meets all of the scientific and socio-economic criteria and is therefore a suitable host country for ICGEB. Up to this point the ideals and the legitimate process of establishing the centre were upheld and selection of the location was to be based on scientific peer reviews through the site visit, a well established and accepted procedure among all in the scientific community.

Tragically, as soon as the results of the scientific peer review was announced, certain parties began to resort to other means in supporting their own candidacies. Political manouvers began to enter the scene. Thus the noble ideals, the sound scientific criteria, the requirement for thorough analysis of financial feasibility were one after the other disregarded with apparent cooperation of some in the UNIDO secretariat. The devotion, the efforts and the recommendations of the Selected Committee of Experts similarly became increasingly disregarded and the mandate of the Belgrade meeting was evaded. With these behind-the-scene manipulations, the Ministerial Level Meeting in Madrid in September 1983, which was supposed to make an easy and legitimate decision based on the recommendation of the Selected Committee of Experts, became instead a political forum *par excellence* to the disillusion and disgust of all scientists present. As a result, again a decision on the location could not be reached. This time a "Preparatory Committee" was formed to work out compromise in order to reach a concensus. During the work of this committee the political element became more and more predominant and was eventually manifested in the form of an imposed decision by a vote totally disregarding the preceeding legitimate mandates, resolution, recommendation and most tragically the shattering effect of such an imposition of will on the good will and the spirit of international cooperation. A substantial proportion of delegations found it unacceptable and declared non-participation in the voting and left the meeting. Regardless of the recognition that such imposition of will was devisive, those parties seeking to imposed political will persisted with unprecedented stubbornness and repeated the damaging acts in a Plenipotentiary Meeting in April 1984 when 12 of the 25 participating delegations either walked out, declared non-participation in voting or voted against the location of ICGEB in Italy and India. None of the countries from Asia and Africa endorsed the locations. With this scenerio and with the facts that none of the most advanced countries in biotechnology, e.g, U.S.A., U.K., France, West Germany, Canada, Japan etc. is likely to participate, the UNIDO's ICGEB is considered dead by scientists witnessing the progression from noble ideals to ugly political manipulations. The most advanced countries were perhaps wise and well experienced with UNIDO and were alerted to the likelihood of such a political imposition and tragic outcome and decided from the onset to stay out.

Together, it was a sad lesson but perhaps an important one if the international scientific community can use this as a case study of how noble ideals and genuine efforts by many can be destroyed by a few who seek to impose their wishes and will on all. This is an important lesson as the need to close that technological gap still need to be met and all should not be discouraged from seeking other mechanisms to bring this important field to the benefit of all developing countries. Any future schemes to be established can gain from the lesson learned in this case study and scientists will presevere and eventually prevail over political lobbyists and truly serve the needies of all nations.

ABSTRACTS

The purpose of this abstracts section is to provide readers of the Asian Pacific Journal of Allergy and Immunology with summaries of original, scientific articles, related to various branches of allergy and immunology, recently published by authors from Thailand, India and China. By this means the editors hope that articles which are highly interesting, but which unfortunately have been lying hidden in less circulated local journals as well as those appearing in better-known journals elsewhere will be collated and will thereby reach readers in various parts of the world. Mechanisms are being worked out to include abstracts of work from other countries of the region.

THAILAND : **S. Vejjajiva, M.D., Editor**

Aspirin-Induced Asthma: A Case Report

M. Tuchinda
Department of Paediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700.
J Paediat Soc Thailand 1983; 22:61-5.

The author describes a case of aspirin-induced asthma in a seven-year-old Thai boy who had suffered from asthma since age of two. The first episode occurred at the age of 3½ years following treatment of an upper respiratory tract infection with medication which included ASA; the severe asthmatic attack which occurred at the time was believed to have been triggered by the viral respiratory tract infection.

Again at seven years of age, another severe asthmatic attack developed shortly after the administration of ASA for pyrexia; a school-teacher had given him the aspirin.

The patient had no evidence of allergic rhinitis, sinusitis or nasal polyps. Allergy skin tests elicited a positive reaction to housedust mite. Other findings were negative. An ASA challenge test was not performed.

The author warned paediatricians to be aware of this condition despite its rarity among children. Patients suspected of ASA sensitivity should avoid not only ASA but also non-steroidal anti-inflammatory drugs and tartrazine.

Immunological Aspects in *Plasmodium falciparum* Infection

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Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok 10400
J Clin Lab Immunol 1983; 12:37.-

Twenty-four patients with *Plasmodium falciparum* were studied. Circulating immune complexes in sera were detected by inhibiting IgG coated latex agglutination with RF factor in 29 per cent of the patients and by EAC rosette inhibition test in 21 per cent. Demonstrated were a low number of T cells identified by E-rosette and high number of B cells which contained immunoglobulins. The authors suggested that T-cell depletion may be caused by antibody to the presence of T-lymphocyte in the sera of these patients. The presence of immune complex in those patients may support the evidence of deposition of antigen-antibody complexes in the kidneys of patients with *P. falciparum* infection.

A Comparative Study of Pregnancy Test Kit Produced in Thailand

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Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400.
J Med Assoc Thailand 1983; 66:227-30.

Indirect haemagglutination inhibition (HI) tests were performed on urinary specimens submitted routinely for pregnancy testing at the Clinical Immunology Laboratory, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital. HI was developed in that laboratory and performed there for 12 years. Qualitative pregnancy tests were performed on 500 specimens and semiquantitative tests on 154 specimens.

The results were compared with a newly developed latex agglutination inhibition test (LI), the so called "Preg test". Both tests gave similar results at HcG levels of < 1 IU/ml and 4-16 IU/ml. There were some discrepancies at the levels of 1 IU/ml and 2 IU/ml. Thirty-two specimens of 1 IU/ml by HI were shown to have mixed results: in 20 specimens the LI test was negative; in 12 specimens, positive. In 50 specimens of 2 IU/ml, the LI test was negative in seven specimens and positive in 43. Although the LI pregnancy test is not trouble-free, the authors claimed it to be as good as the commercial products from other countries.

TORCHES Agents as Possible Causes of Spontaneous Abortion in Thai Women

S. Tantivanich, S. Tharavanij
Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400.
J Med Assoc Thailand 1983; 66:443-7.

Sera collected at Phra Mongkutklao Hospital from 100 normal pregnant women and 31 women who had aborted were

tested for IgG and IgM ELISA antibodies against rubella, CMV, HVH, *Toxoplasma* and syphilis. The normal pregnant women did not have IgM antibodies; however, IgG antibodies against rubella, CMV, *Toxoplasma*, syphilis, HVH1, HVH2, HVH1 and HVH 2 were detected in 19 per cent, 3 per cent, 1 per cent, 2 per cent, 4 per cent, 12 per cent and 2 per cent, respectively, of the women tested.

In the women who aborted, IgM antibodies against TORCHES agents were positive against HVH (38.7%), rubella (9.6%) and CMV (3.2%). The remaining women who had aborted showed mixed IgM antibodies against rubella and HVH (9.6%); CMV, *Toxoplasma* and HVH (3.2%); CMV, rubella and HVH (3.2%); and *Toxoplasma* and HVH (3.2%). These results strongly suggest that HVH infection is the major causative agent of abortion in Thailand or that it is closely related to abortion followed by rubella and CMV infection, respectively.

Immunopathology of Acute Rheumatic Fever and Rheumatic Heart Disease. The Demonstration of Coxsackie Group B Viral Antigen in the Myocardium

B. Pongpanich, S. Boonpucknavig, C. Wasi, P. Tanphaichitr, V. Boonpucknavig

Departments of Paediatrics and Pathology, Faculty of Medicine, Ramathibodi Hospital; Department of Pathobiology, Faculty of Science; and Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok.

Clin Rheumatology 1983; 2:217-22.

Immunopathological studies were performed on the heart tissue of 12 patients with both acute rheumatic fever with carditis and chronic rheumatic heart disease. Coxsackie group B viral antigen was demonstrated in three heart specimens of patients clinically compatible with active rheumatic fever. In two of these, the pathological findings were compatible with acute rheumatic carditis. The serum for neutralising antibodies to Coxsackie B 4 was 1:40 in one of the three cases, which occurrence was compatible with a recent infection in that patient. Immunoglobulin was detected in two and complement in one of the heart specimens. All of the chronic rheumatic heart specimens and the controls were negative. These findings suggested that Coxsackie group B virus may be aetiologically related to the pathogenesis of acute rheumatic fever.

Renal Histopathology in the Haemolytic-Uraemic Syndrome Following Shigellosis

F.T. Koster, V. Boonpucknavig, S. Sujaho, R.H. Gilman, M. Mujibar Rahaman

Johns Hopkins University International Center for Medical Research, and Cholera Research Laboratory (ICDDR, B), Dacca, Bangladesh, and Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok.

Clin Nephrology 1984; 21:126-33.

The haemolytic-uraemic syndrome (HUS) following dysentery caused by *S. dysenteriae* Type 1, characterised by microangiopathic haemolytic anaemia and acute renal insufficiency, is clinically similar but not identical to the idiopathic HUS. We studied renal necropsy specimens of nine children who died from HUS following shigellosis. Using light and immunofluorescent microscopy, we compared them with 12 controls: six cases with severe shigellosis without HUS, and six with pneumonia or sepsis. Eight of nine HUS cases showed cortical necrosis, extensive glomerular thrombosis or arterial thrombosis. Cases without HUS showed only scattered glomerular fibrin, thrombin and widening of the mesangium. Among seven HUS cases studied by immunofluorescent microscopy, three demonstrated deposition of glomerular IgM and complement (C3) and one of the three had IgG and IgA as well; four cases had neither immunoglobulin or complement deposits. Among nine controls, two demonstrated IgM and three IgG but none had C3. Both HUS and non-HUS cases had fibrin deposition. In the three HUS cases studied by electron microscopy, intracapillary material (fibrin and platelets) was seen in all three, and sparse electron-dense deposits were seen in the mesangial matrix in one. The data indicate that the renal histopathology in HUS following shigellosis consistently presents symptoms of severe thrombotic microangiopathy, but lacks the characteristic endothelial and mesangial lesions of idiopathic HUS. The infrequent demonstration of glomerular immunoglobulin deposition fails to support an immunoglobulin-mediated pathogenesis.

INDIA : L.M. Srivastava, M.D., Ph.D., Editor

Serum Mediated Suppression of Mitogenic Responses in Leprosy and Reversal by Foetal Calf Serum

V.L. Jaswaney, J.S. Nadkarni, S.S. Rao

Institute for Research in Reproduction, and Tata Memorial Centre, Bombay, India

Ind J Exp Biol 1983; 21:59-61.

Impaired lymphocyte reactivity to PHA was observed in untreated lepromatous leprosy (LL) patients. A significant reduction of mitogenic response was observed when peripheral blood lymphocytes (PBL) from normal subjects were incubated in the sera of LL patients. However, sera from tuberculoid leprosy (TL) patients showed only partial suppression. The mitogenic response of PBL from LL patients was restored to nearly normal levels when they were incubated for 18-24 hours in a medium containing 20% foetal calf serum (FCS). Incubation in a medium with human AB sera failed to bring about any such restoration. The cell-free supernatant of the cultures containing FCS but not AB sera suppressed the mitogenic response of normal T cells.

Use of Immunofluorescence Technique to Demonstrate Complement Activation *In Vitro* by Microbes

O.M. Prakash, V.D. Ramanathan, V. S. Gupta
Central Jalma Institute for Leprosy, Taj Ganj, Agra, India.
Ind J Exp Biol 1983; 21:674-6.

A fluorescence assay for detecting complement activation *in vitro* by microbes at the C_3 level was described and compared with conventional two-dimensional immunoelectrophoresis.

Role of Accessory Cells in *In Vitro* Stimulation of Rabbit Lymphocytes by Herpes Simplex Antigens: Evidence for Proliferation of DH Cells

A.K. Kapoor, I.V. Khan
Department of Experimental Pathology, the Medical School, Vincent Drive, Birmingham B 152TJ, England
Ind J Exp Biol 1983; 21:294-6.

The study demonstrates the requirement of phagocytic as well as of surface adherent cells in *in vitro* immune specific stimulation of rabbit blood lymphocytes to HSV type I antigens. On the contrary, surface-adherent cells do not appear to be important with regard to herpes-specific activation of lymph node cells (LNC). These LNC appear to contain large numbers of antigen-reactive lymphocytes which may be sufficient to initiate lymphoproliferative response without macrophage help.

Circulating Immune Complexes in Glomerulonephritis with Special Reference to Minimal Disease

R.L. Mehta, N.K. Ganguly, S. Seghal, P.C. Singhal, K.S. Chugh
Departments of Nephrology, Microbiology and Immunology, Postgraduate Institute of Medical Education and Research, Chandigarh, India
Ind J Med Res 1983; 77:96-106.

Circulating immune complexes (CIC) were determined in 32 cases of glomerulonephritis and 32 normal controls utilising C_1q-binding solid-phase assay techniques. Significantly high levels of CIC were detected in 61.9 per cent of cases with minimal change glomerulonephritis and the majority of patients with membranoproliferative lesions, diffuse proliferative glomerulonephritis, focal glomerulosclerosis and chronic slcerosing glomerulosclerosis ($p < 0.001$). Low levels of complement as well as positive immunofluorescence in the glomerular tuft were shown in 71.4 per cent of the cases of minimal change glomerulonephritis. These findings suggest that minimal change glomerulonephritis may be an immune complex disorder in which the immune complexes play a significant role by interacting with cellular receptors and releasing mediators by interfering with the helper or suppressor functions without accumulating in the glomerular tuft in all patients.

Active and Total E-Rosette Forming T-Lymphocytes in Pulmonary Tuberculosis

T. Prabhu, V. Reddy
National Institute of Virology, Pune, and Department of Paediatrics, Pandit Jawaharlal Nehru Medical College, Raipur, India
Ind J Med Res 1983; 77:308-13.

Active and total E-rosette-forming cells (A-RFC and T-RFC) were estimated in the peripheral blood of pulmonary tuberculosis patients with active disease, of relapsed cases, and treated cases. It was observed that in active cases A-RFC and T-RFC levels were decreased whereas in relapsed patients A-RFC levels were significantly reduced; the T-RFC levels were similar to the percentage observed in controls. Treated patients as well as controls had higher levels of both cell types. In patients who showed improvement after two months of treatment, there was a concomitant increase in the number of both cell types. However, in all types of patients A-RFC appeared to be the more significant subpopulation of T-lymphocytes. There was no relationship between the intensity of delayed hypersensitivity and the levels of A-RFC and/or T-RFC.

Alterations in Leukocyte Rhythm in Microfilaraemic Subjects – A Preliminary Report

V.K. Srivastava, R. Chandra, J.C. Katiyar, P.K. Murthy, P.A. George, A.B. Sen
Upgraded Department of Social and Preventive Medicine, K.G. Medical College, and Divisions of Parasitology and Biometry and Statistics, CDRI, Lucknow, India
Ind J Med Res 1983; 77:314-7.

Leukocyte rhythm in microfilaria-positive subjects in an endemic village was studied. In normal healthy subjects, there was a sharp fall in the number of leukocytes after attaining maximum counts, whereas in microfilaria-positive cases, the fall was gradual almost coinciding with the decline in microfilaria counts. The significance of elevated levels of leukocytes in the presence of microfilaria in circulating blood is discussed.

Total Serum Immunoglobulins in Japanese Encephalitis Patients with High IgE Levels in Acute Phase

H. Bashir, K.M. Shaikh, C. Pavri, L. Ramamoorthy, S.P. Verma, N.J. Deuskar
National Institute of Virology, Pune, India
Ind J Med Res 1983; 77:765-9.

Total serum immunolobulins, viz., IgM, IgG and IgA, were determined using the same serum samples which, in an earlier study, had been shown to have high levels of total serum IgE during the acute phase of Japanese encephalitis. Statistical analysis of the results showed that consistently significant differences were observed only with IgE and not with the other immunoglobulins.

Counter-immunoelectrophoresis for *Salmonella typhi*-Antigen and Antibody Detection in Typhoid

B.N. Harish, R.S. Rao
Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research, Pondichery, India
Ind J Med Res 1983; 77:781-4.

Counter-immunoelectrophoresis (CIE) was used for the simultaneous detection of *Salmonella typhi* antigen and antibody in the sera of patients with typhoid fever using a hyperimmune *S. typhi* antiserum and the sonicate of the organism. Antigen was detected in 16 of 28 patients (56%) with positive blood cultures. It was not detected in any of the 47 patients with negative blood cultures but positive Widal tests or in 163 control sera. Antibody was detected in 12 of the 28 (44%) positive blood cultures and in 34 of the 47 (72%) Widal-positive sera. Precipitating antibody was detected when agglutinin levels were < 160. All the patients with positive blood cultures were positive for either antigen or antibody. There were no false positive reactions. The CIE test was found to be specific for typhoid and appears to hold promise as a rapid diagnostic test.

Immune Complexes and Immunoglobulins Involved in Human Filariasis

G.B.K.S. Prasad, M.V.R. Reddy, B.C. Harinath
Department of Biochemistry, Mahatma Gandhi Institute of Medical Sciences, Sevagram, India
Ind J Med Res 1983; 77:813-6.

Circulating immune complexes (CIC) were determined by using 3% PEG precipitation and complement- consumption tests in 40 sera of filarial patients with either microfilaraemia or clinical manifestations such as elephantiasis, hydrocoele etc, and 15 healthy persons. Significantly elevated levels of CIC were observed in cases of clinical filariasis compared with microfilaraemia and endemic normals. Immunoglobulin classes involved in immune complexes in clinical filariasis were detected by immunodiffusion and immunofluroscence assays; IgG and IgM were mostly found to be present in immune complexes.

Detection and Monitoring of Microfilarial ES Antigen Levels by Inhibition ELISA During DEC Therapy

A. Malhotra, B.C. Harinath
Department of Biochemistry, Mahatma Gandhi Institute of Medical Sciences, Wardha, India
Ind J Med Res 1984; 79:194-8.

Inhibition ELISA for the detection of *Wuchereria bancrofti* microfilarial ES antigen has been described. Fifteen of 20 microfilaraemic patients, seven of 20 cases of clinical filariasis, four of 20 endemic normals and none of the seven nonendemic normals showed the presence of filarial antigen by inhibition ELISA. The antigen titres during DEC therapy in 10 microfilarial carriers showed an initial increase followed by a gradual decrease during treatment.

Immune Complex Mediated Cardiac Lesions in Rats

V. Nagal, N.K. Ganguly, R.N. Chakravarti
Department of Experimental Medicine, Postgraduate Institute of Medical Education and Research, Chandigarh, India
Ind J Med Res 1984; 79:250-9.

The effects of bovine serum albumin (BSA), its antibody and preformed immune complexes (BSA-anti-BSA) on the production of cardiac and renal lesions in normal and hypercholesterolaemic rats have been studied. Following a high fat diet for a period of one month, significant hypercholesterolaemia was produced. Hyperlipaemic rats given immune complex injections showed precipitin lines against antigen and antibody in most of the rats. The percentage binding of CIC was of a moderate order and varied from 4 per cent to 10 per cent. The complement pool size was greatly reduced. Various degrees of myocardial degeneration were seen among hypercholesterolaemic rats that had been given immune complexes. One animal of this group had extensive aortitis. Microscopic examination of kidneys revealed morphological features of immune complex glomerulonephritis in both normal and hyperlipaemic rats.

Beclomethasone Dipropionate and Betamethasone Valerate with Sodium Cromoglycate in Steroid-Dependent Asthma in Children

S.K. Gupta, S. Chatterjee, K. Mitra
Department of Respiratory Diseases, the Calcutta Medical Research Institute, and the Institute for Respiratory Diseases, Calcutta
JAPI 1984; 32:245-9.

In a three-year study of 32 children with steroid-dependent extrinsic asthma and its complications, only 24 (75%) could be completely removed from oral steroid therapy on a pre-determined schedule of a combination of beclomethasone dipropionate or betamethasone valerate with sodium cromoglycate, long-acting ACTH and an oxygen-driven aerosol of L-acetylcysteine and salbutamol. Three patients (9%) were regarded as "failure cases" while five patients (16%) had to be given steroids intermittently (3-8 times a year) to control acute bouts of spasm and were regarded as being a "partial success".

This report contradicts some of the published reports regarding oral steroids vis-a-vis steroid aerosols.

CHINA : S.T. Ye, M.D., and A.R. Wu, M.D., Editors

Scanning Electron-Microscopy on Artemisia Pollen: An Important Allergenic Pollen in Northern China

S.T. Ye, B.S. Qiao, Y.J. Lu, X.Q. Li
Department of Allergy, Capital Hospital, Beijing
Chinese J Otorhinolaryngol 1983; 18:214-5.

Artemisia pollen has been confirmed as an important allergen in northern China since the end of the 1950s. Scanning electron-microscopic examination of *Artemisia annua* and *Artemisia sieversiana* was performed in our laboratory during the past two years. Fifteen scanning pictures are presented in this paper. In comparison with photomicroscopy, some morphological revisions were made on the basis of electron-microscopy as follows : 1) Both the *A. annua* and *A. sieversiana* pollens are oval longitudinally instead of spherical as previously recognised using photomicroscopy; 2) by direct microscale measurement of 159 pollen grains, the average size of *A. annua* and *A. sieversiana* was found to be 19.4 x 13.1 μ and 20.2 x 13.8 μ respectively. They are actually smaller than measured previously by photomicroscopy; 3) there are numerous blunt rudimentary spines scattered all over the exine of the pollen surface; 4) due to the deep concavity of the furrows on the pollen, not a single pore of the pollen could be visualized by scanning electron-microscopy: the pores were hidden by the edges of the furrows; and 5) in some of the pollen grains, several holes or warty growths from 0.5 to 2 μ in diameter were observed at the surface of the pollens; they are considered to be developmental defects.

Bronchial Provocation Test with *Artemisia* Pollen Antigen

Y.Q. Pan, S.T. Ye
Department of Allergy, Capital Hospital, Beijing
Chinese J Tuberc Respir Dis 1983; 6:138-40.

A bronchial provocation test procedure using *Artemisia* pollen extract was recommended for the specific diagnosis of an autumnal type hay-fever and asthma induced by *Artemisia* pollen. Seventy-six per cent of a total of 41 cases, all with a positive reaction to *Artemisia* pollen extract using intracutaneous tests, gave a positive reaction upon bronchial provocation with the same allergen. A fall of FEV_1 (up to 15%) was considered to be the minimal margin for positive provocation reaction. Wheezing rales spread from the upper lobe of each lung to the whole lung field following the challenge. Control tests on 30 healthy subjects were all negative. Eosinophil examination of 15 sputum specimens collected immediately after positive provocation showed 14 positive specimens. These data indicate that the bronchial provocation test with *Artemisia* antigen is specific. The recommended procedure is considered to be safe and highly reproducible owing to the strictly controlled dosage of allergen inhaled and the close observation of the reaction.

Aspirin-Induced Asthma and Aspirin Triad (Analysis of 30 Cases)

H.Y. Zhang, S.T. Ye
Department of Allergy, Capital Hospital, Beijing
Chinese J Tuberc Respir Dis 1983; 6:146-8.

Thirty cases of aspirin-induced asthma were reported. All had severe asthmatic attacks after a "regular" dose of aspirin. Sixteen of the 30 cases showed a typical aspirin triad; eight showed a mild skin reaction to common inhalant allergens; and 12 required long-term use of corticosteroids for symptomatic control. Pyramidon, paracetamol, analgin and indomethacin also provoked attacks of asthma in some of the cases. Oral betamethasone and regular inhalation of beclomethasone dipropionate were very successful in bringing about a subsidence in asthmatic attacks in our patients.

The Correlation of a Bronchial Provocation Test, Intracutaneous Test with *Artemisia* Pollen Antigen and Serum-Specific IgE Levels

Y.Q. Pan, H.J. He, S.Q. Duan, S.T. Ye, J.F. Song, M.Y. Fong, A.R. Wu
Department of Allergy, Capital Hospital, and W.H.O. Collaborating Centre for Research and Training in Immunology, Institute of Basic Medical Sciences, Beijing
Chinese J Microbiol Immunol 1983; 3:288-92.

The results of bronchial provocation tests, intracutaneous tests with *Artemisia* pollen extract and serum-specific IgE levels measured by ELISA were compared for a group of asthmatic patients. Out of 41 cases with positive intracutaneous tests, 31 (76%) showed a positive reaction to bronchial provocation tests. Nineteen out of 25 patients (76%) who were positive to bronchial provocation, exhibited positive serum-specific IgE levels. Out of 28 patients with positive specific IgE measurement, 19 (68%) showed positive reactions to bronchial provocation tests. Based on the results of this comparison, it seems that the degree of bronchial sensitivity cannot be predicted by the degree of skin reactivity and serum specific-IgE levels. Several factors influencing the correlation of these tests were discussed.

Identification of Anti-idiotypes and Their Antibodies

B. Lu, P.O. Couraud, A.D. Strosberg
Institute of Basic Medical Sciences, Academy of Military Medical Sciences, Beijing, and Laboratory of Molecular Immunology, IRBM, CNRS – University of Paris 7, Paris.
Chinese J Microbiol Immunol 1984; 4:6-10.

Anti-idiotypic antibodies were raised against anti-catecholamine ligand antibodies. The anti-iditoypic response was shown to be cyclical and to correspond to the production of antibodies which could bind to catecholamine β-adrenergic receptors

and stimulate adenylate cyclase. Disappearance of these antibodies from the serum could be correlated with the appearance of a catecholamine ligand binding activity corresponding to the synthesis of autologous anti-idiotypic antibodies directed against the induced anti-id molecules. Comparison of the injected versus the induced anti-ligand antibodies revealed striking differences in affinity but similarity in the ability to bind the anti-idiotypic antibodies and to the ligand containing affinity gel. The results support the existence of a functional network of idiotype and anti-idiotype interaction involving external as well as internal antigens, antibodies and other types of molecules involved in recognition phenomena, such as hormone receptors.

The Role of Macrophages in Experimental Anti-leptospiral Infection

J. Fan, Ch. Yao
Institute of Epidemiology and Microbiology, Chinese Academy of Medical Sciences, Beijing.
Chinese J Microbiol Immunol 1984; 4:17-21.

The role of macrophages in experimental leptospiral infection in mice was studied. Peritoneal macrophages taken from immunised mice with heat-killed leptospira were transferred to normal mice treated with cyclophosphamide (300 mg/kg) 48 h earlier, and such mice showed specific protection against leptospira infection. When macrophages were damaged with SiO_2, the level of antibody was decreased. Immune macrophages incubated with leptospira in vitro showed cytotoxic effects against leptospira. Acid phosphatase of macrophages was determined, and some significant differences were found between the functional activity of peritoneal macrophages taken from immunised versus normal mice, indicating that acid phosphatase played a role in the interaction of macrophages with leptospira. Our experimental results showed that both humoral and cellular immunity are important for resistance against leptospiral infection.

Application of Single Radial Hemolysis Test for the Detection of Antibodies Against Dengue and other Togaviruses

X. Tian, Y. Wen, H. Xu, Ch. Ai
Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, Beijing.
Chinese J Microbiol Immunol 1984; 4:40-2.

Experiments were made to detect antibodies against dengue, Japanese B encephilitis (JBE) and other togaviruses by the single radial haemolysis (SRH) test. We tested 337 serum samples from healthy individuals on Hainan Island, where dengue epidemics were reported in 1980, twelve serum samples from convalescent patients with JBE, and immune mouse ascitic fluids against dengue 1-4, JBE, West Nile and Chikungunya viruses. Sera of healthy people from a nonepidemic area were tested as controls. The results showed that this technique is as sensitive as the conventional haemagglutination inhibition test (HIT), but more specific than the HIT, and its reproducibility is satisfactory. It is easy to perform and therefore very useful in large-scale sero-epidemiologic studies of togaviruses.

Study on the Rapid Detection of Dengue Virus by SPA Co-agglutination

R. Chen
Institute of Military Medicine PLA, Guangzhou Unit, Guangzhou.
Chinese J Microbiol Immunol 1984; 4:43-4.

The co-agglutination test were carried out with *Staphylococcus aureus* protein A-rich strain coated by anti-arbovirus immuno-ascites fluid for the detection of dengue virus type 1-4, Japanese B, Chikungunya, yellow fever (17D strain) and West Nile virus antigens. Positive reactions occurred only for specific virus antigens, and were visible to the naked eye within 1-3 minutes. The co-agglutination titre was as high as 1:40 to 1:1600. Its specificity was comparable to that of the complement fixation test and its sensitivity was approximately the same as that of the haemagglutination test. In typing dengue virus strains isolated from patients, the results of co-agglutination tests were identical to those of the complement fixation and neutralisation tests, and well correlated with that of TCD_{50} using mosquitoes. It is obvious that the co-agglutination test is a specific, sensitive, easy to perform and rapid diagnostic method.

Detection of Epstein-Barr Virus IgA/EA Antibody for Diagnosis of Nasopharyngeal Carcinoma by Immunoautoradiography

Y. Zeng, C. Gong, M. Jiang, L. Zhang, Zh. Fang
Institute of Virology, Chinese Academy of Medical Sciences, Beijing, and Wuzhou Cancer Research Unit, Wuzhou.
Chinese J Microbiol Immunol 1984; 4:45-7.

An immunoautoradiographic method (IR) was used for detection of EB virus IgA/EA antibody in sera from a nasopharyngeal carcinoma (NPC) patient and from other control groups. Ninty-six per cent of NPC patients had IgA/EA antibodies with a high titre of GMT. The positive rate and GMT of IgA/EA antibodies in patients with malignant tumours other than NPC and in normal individuals were only 4 per cent and 0 per cent respectively. Fourteen NPC patients had no IgA/EA antibody detected by the immunofluorescence test (IF) and immunoenzymatic test (IE), but 11 of them had IgA/VCA and six had IgA/EA antibodies by IR. These data indicate that IR is more sensitive than IF and IE for detection of IgA/EA antibody and may be used for detection of NPC.

Regulation of IgE Antibody Production in Mice. I. IgE and IgG Antibody Responses in Different Strains of Mice to Trichsanhes Kirilowii Maxim (TKM)

Sh. Zheng, H. Liu, Y. Li, Y. Zhang, Y. Yu, A.R. Wu
Department of Immunology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, and WHO Collaborating Center for Research and Training in Immunology, Beijing.
Chinese J Microbiol Immunol 1984; 4:50-4.

The purpose of this study was to investigate and compare the production of specific IgE and IgG against TKM in 8 inbred strains of mice when the antigen was injected with Al $(OH)_3$ or complete Freund's adjuvant (CFA). Specific IgE and IgG antibodies were detected by passive cutaneous anaphylaxis (PCA) in rats and by ELISA, respectively. The results showed that TKM-specific IgE and IgG antibodies could be detected in all 8 strains of mice after a single injection of 1 μg or 5 μg of TKM when $Al(OH)_3$ was used as an adjuvant, and good secondary responses were obtained after a booster. In contrast to $Al(OH)_3$, CFA showed poor ability for eliciting IgE antibody in 1-μg TKM groups even though that is enough for stimulating specific IgG production. However, in 5-μg TKM groups, in addition to IgG antibody, low level IgE antibody appeared in C3H/H3-mg, C3H, C57BL and DBA/2 strains after booster injection, and among them, only the C3H/He-mg and C57BL strains produced IgE antibodies during primary response. IgE responses in different sexes were also studied. Among four strains studied, only in Swiss mice did one sex (the females) demonstrate a higher IgE response than the other. Based on the magnitude of IgE response to 1 μg TKM with Al $(OH)_3$, according to our own criterion, the 8 inbred strains of mice can be divided into 3 groups: C3H and C3H/He-mg were ranked as high responders, C57BL and Swiss as low responders, and the others in between.

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7. Agency Publication
   National Center for Health Statistics. Acute conditions: incidence and associated disability, United States July 1968-1969. Rockville, Md.: National Center for Health Statistics, 1972. (Vital and health statistics. Series 10: Data from the National Health Survey, no. 69) (DHEW publication no. [HSM] 72-1036).

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A Malaria Vaccine–Necessary But Not Sufficient

In 1983 there were 220 million cases of malaria world-wide – mostly caused by *Plasmodium falciparum*. In tropical Africa alone there were one million malaria deaths among children. At the present time 2 billion people live at risk in malarious areas of the world. Malaria is without doubt the most destructive of infectious diseases and the harm it does is not only measured in cost to human life but also in the destruction of human potential.

During the 1970s the World Health Organisation's strategy for the eradication of malaria began to deteriorate. National malaria programmes mounted in the 1950s and 1960s were based on the availability of new and effective residual insecticides for the control of the mosquito vector and antimalarial drugs for prophalaxis and treatment of infection. Implementation of these programmes required major resource and financial expenditures. Erosion of these efforts came about basically in three ways. First, partial successes in the eradication strategy produced a false optimism which reduced national health priorities and expenditures for malaria control. Second, the anopheline mosquito began to develop resistance to residual insecticides. Third, the major human malaria pathogen, *Plasmodium falciparum*, developed resistance at an alarming pace to the available armamentarium of antimalarial drugs. These factors and others have combined to produce a situation today that WHO characterises as stagnant.[1]

It is, therefore, no surprise that the current prospect for the development of a malaria vaccine has been received with much hope and enthusiasm. The idea of a malaria vaccine is of course not new, but since about 1980 a critical effort has been underway to field a product for human use. Work on developing a vaccine has been spurred by major scientific advances occuring throughout the 1970s – hybridoma techniques for producing monoclonal antibodies, recombinant DNA methodology essential to gene cloning and, in the specific case of the malaria parasite, the technique for cultivating *P. falciparum*.

Work on developing a vaccine has progressed along three lines of attack on the complex malaria life-cycle targeting specifically the sporozoite, blood stage and gamete forms. The most promising development has been towards the production of a sporozoite vaccine. The prospect for a vaccine against the gamete is also good. Identification of the relevant blood stage antigens has proven more difficult, although a number of candidate antigens are under intensive study.

Malariologists have, however, recognised that there are practical problems involved with a vaccine directed at only one point in the malaria organism's life-cycle. A sporozoite vaccine must have an "all-or-none effect," since a single surviving sporozoite reaching the host's liver has the potential for causing infection. A gamete vaccine which would act to block transmission of infection is at best a tool for use in the long-range control of malaria. In the case of a blood stage vaccine, infection would not be prevented but attenuation of clinical symptoms would be achieved. Because of these individual vaccine considerations, there is a consensus for a combination or polyvalent vaccine aimed at all three stages in the life cycle of *P. falciparum*.[2]

Even if the ideal of a polyvalent malaria vaccine is achieved, there will be, nonetheless, serious problems with its practical application. The issue is whether a malaria vaccine will be effective in a malaria endemic population. For nonimmune individuals even a monovalent vaccine may provide limited but useful protection, but for a malaria endemic population it is questionable whether immunisation can be achieved in a majority of individuals.

Under natural conditions protective immunity to infection with *P. falciparum* is poorly acquired, often incomplete and transient. Immunosuppression is a major feature of falciparum malaria and immunisation even with heterologous antigens can be difficult. In addition the parasite may introduce complications through antigenic heterogeneity or other mechanisms for eva-

ding host defences.

Success against these rather formidable problems regarding a malaria vaccine will only be achieved through a long process of field testing and refinement. The message here is that while the development of a prototype malaria vaccine may be imminent, an efficacious vaccine for most of the world's at-risk population may be expected at best in the distant future.

We must not, therefore, allow our enthusiasm for a vaccine solution to the malaria problem to obscure the immediate and near future realities of malaria's actual and potential destructive force. The lessons of past failures in our struggle against malaria should teach us that we must bring to bear on malaria all the weapons we can muster. This means not only a vaccine but innovative means of vector control and new and noval chemotherapeutic agents.

In particular we need new antimalarial drugs. It must be recognised that we have reached the point where *P. falciparum* has demonstrated resistance to all the clinically proven drugs – including the newest, mefloquine. What should be most disturbing is that after mefloquine there are only a few candidate drugs under development and most of these are structually related to mefloquine and thus are potentially subject to cross-resistance. So there is a very real possibility that in 5-10 years we may have exhausted our chemotherapeutic resources against malaria.

Thus it should be obvious that concurrent with vaccine development there should be a programme for the production of antimalarial drugs. Yet during the past 20 years there has been only one sustained effort for antimalarial drug development: the US Army's Walter Reed programme which produced mefloquine. The basic approach in this programme is empirical search for lead compounds which are then rationally developed. Given the current malaria situation this approach cannot be counted upon to produce the diversity and number of drugs needed. One or two more drugs in the next 20 years – even if achieved by empirical search – will not solve the problem. What is needed is a truly rational approach to the development of antimalarial drugs.[3] By rational is meant the design of agents based on a detailed understanding of the malaria parasite's biochemistry whereby parasite-unique metabolic features are selected for drug targeting. In addition, biochemical targeting and agent design must in the case of malaria be complemented by an understanding of the molecular basis of resistance to current drugs.

The new biotechnology provides a powerful tool with which to pursue the objective of rational drug development. What is needed is a "critical mass" of scientific talent sensitised to the unique problems of malaria drug development with long-range institutional support and international coordiation.

A rational approach to antimalarial drug development is an achievable goal. But it is a costly and demanding one and therefore must compete with other approaches such as vaccine development. At the current time a first generation malaria vaccine may be a soon-to-be achievement. This is not the case for a rationally developed new antimalarial drug. It is a matter of concern that the prospect of a vaccine will devalue the emphasis and hence support for drug development. We must not let this happen. For, while a malaria vaccine is necessary, it is not alone sufficient to control and eventually eliminate the world malaria problem.

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REFERENCES

1. World Health Organization Report of the Steering Committees of the Scientific Working Groups on Malaria (June 1980-June 1983).
2. Wernsdorfer WH. Prospects for the development of malaria vaccines. Bull Wld Hlth Org 1981;59:335-41.
3. Webster HK. Antimalaria drug resistance: problems and prospects. Thai J Pharmacol 1982;4:171-91.

The Current Clinical Value of Broncho-alveolar Lavage: A Personal View

Broncho-alveolar lavage was first introduced in the mid-1960s to remove tenacious secretions from patients with asthma and cystic fibrosis; it was also developed into a procedure of whole lung lavage in the treatment of alveolar proteinosis.[1]

It was reintroduced in the late 1960s and early 1970s in an attempt to identify cells and products from the lungs that could not be sampled from the blood. The procedure has provided a large amount of exciting material for research fundamental to chronic inflammation at tissue sites and has given much insight about the interaction of individual cell types, since lavage cells are living and can be maintained in tissue culture over considerable periods of time. Lavage has been especially exciting as a tool to evaluate the function and morphology of living alveolar macrophages because these possess properties different from monocytes and cannot, of course, be studied from blood samples.

Some claims have been made for the value of lavage in a clinical context. Some of these have been fulfilled and others not. The purpose of this editorial is to present a personal view of the current position, recognising that it is not possible to do full justice to all the work undertaken, some of which presents conflicting results. Nor is it intended as a final judgement, for much more work still needs to be done.

What are the questions?

The question of what is the "clinical value" of lavage must be stated more precisely because 'clinical value' can be defined under at least three headings:

1. The diagnostic value.
2. The value in indicating the need to initiate therapy.
3. The value in monitoring activity in the titration of drugs during the course of therapy.

Diagnosis

Lavage is of considerable value in diagnosis provided that the specimens are properly evaluated by experienced pathologists and prepared and stained appropriately. Lavage has two orders of diagnostic value: first, through its pathognomonic appearances and, second, by differential counts, suggesting a priority of more probable diagnoses without implying absolute specificity.

Some of the pathognomonic appearances include those of malignant cells in lung cancer or lymphomas; the presence of Langerhans' cells on electron microscopy in eosinophilic granuloma; the presence of iron-laden macrophages in idiopathic pulmonary haemosiderosis; the presence of asbestos bodies in those exposed to asbestos, and other identifiable materials encountered in specific occupations such as those using silica, talc and hard metals. Microprobe analysis will identify these specifically, but the characteristics of the material are often suggested in a routine preparation or under polarised light.

Extracellular eosinophilic material, highly suggestive of alveolar proteinosis, may establish a rare diagnosis, important because of its specific treatment. Other features may strongly support this diagnosis. Electron microscopy will confirm the presence of the whorled lamellar bodies, so characteristic of this condition. In some cases, even the macroscopic appearance of lavage fluid is almost diagnostic and "milky" opaque fluid is highly suggestive of alveolar proteinosis, even on naked eye examination.

Examination of lavage fluid can be extremely valuable in the diagnosis of infection, especially by opportunist organisms, provided that specimens are stained correctly. This usually requires an appropriate index of suspicion by the physician before lavage is undertaken, so that the pathologists may be alerted to process the specimens optimally. For example, *Pneumocystis carinii* cysts can frequently be found if silver stains are used; fungal mycelia may be similarly identified. Cytomegalovirus inclusions may be seen with Giemsa's stains, suggesting this type of specific pneumonia. Culture for bacteria, including *M. tuberculosis*, may yield valuable diagnostic information. Because unusual pneu-

monias are often important, it is wise to include a wide range of culture media appropriate for acid fast bacilli, fungi, *Actinomyces* and *Nocardia*, as well as more common organisms.

Differential counts of inflammatory cells, although far from specific, may have diagnostic value in suggesting a priority of likely possibilities in interstitial lung disease. For example, very high lymphocyte counts of greater than 60 per cent are more likely to indicate a hypersensitivity pneumonitis or tuberculosis than sarcoidosis and one or other of these conditions is far more likely than that of cryptogenic fibrosing alveolitis (synonym: idiopathic pulmonary fibrosis), which very rarely produces a lymphocyte count of this order. On the other hand, high neutrophil counts of greater than 50 per cent may suggest a bronchial component to the lavage material or the presence of a bacterial infection. Moderately raised neutrophil counts are common in fibrosing alveolitis and may occur in the late fibrotic stages of any form of interstitial lung disease, including asbestosis, fibrotic sarcoidosis and late-stage hypersensitivity pneumonitis.

A cell profile may give more information than increases in a single cell type. For example, lymphocytes, together with mast cells, are more characteristic of hypersensitivity pneumonitis than sarcoidosis. The frequency of atypical "activated" lymphocyte is also often greater in hypersensitivity pneumonitis than in sarcoidosis, although occurring in both.

An increase in neutrophils, together with eosinophils, is more characteristic of cryptogenic fibrosing alveolitis with or without associated connective tissue disorders than asbestosis but, in the latter, large numbers of asbestos bodies are almost invariably seen.

Thus, differential counts, interpreted by an experienced pathologist with good preparations, can be of great value. Poor preparations, preparations, viewed by someone unfamiliar with the subtleties of lavage samples, will certainly not provide optimal results and may lead to misleading reports being issued. In particular cell distortion can lead to other cell types, especially macrophages, being counted as lymphocytes with obvious misleading implications.

Sporadic ill-considered lavages, performed and interpeted by non-experts, should not be undertaken. Under these circumstances, it is probably wiser to remain on more secure ground using lung biopsies, remembering that small tissue samples of transbronchial biopsies are, themselves, often misleading in interstitial lung disease, and particularly when examined by a non-specialist pulmonary pathologist. Our current preference is for open biopsy in the diagnosis and staging of interstitial disease, where pathognomonic appearances are less likely, and where other diagnostic procedures have failed.

Lavage as an indicator to initiate therapy

It has been suggested that a high lymphocyte count (greater than 28 per cent T cells) in sarcoidosis[2] indicates the need to start steroid therapy. The basis for this is that a group of patients with high counts showed a greater clinical deterioration over six months compared with those having fewer T lymphocytes. Before this policy is adopted two additional facts need to be considered. First, about 70 per cent of patients with sarcoidosis having parenchymal shadows will resolve spontaneously without treatment. This implies that they may pass through a phase of some physiological and radiographic deterioration but still remit spontaneously and, in the view of many physicians, do not require treatment. High lympocyte counts under these circumstances do not necessarily imply *continuing* progressive disease in the longer term. Second, many patients with more persistent chronic sarcoidosis have normal or only slightly raised lymphocyte counts, but will respond substantially to treatment. To deny these patients the chance of improvement on the basis of the lymphocyte count would not seem to be wise. Absolute statements about which patients require and do not require treatment with steroids are, however, not yet agreed upon and there is a very wide variety of physicians' opinion. Until this metter is clarified, it is wise not to rely on a lymphocyte count alone, which has clear logical and factual limitations, as summarised above. The overall state of the individual patient, using the clinical, physiological and radiographic state and the progress over time, are certainly as important, and perhaps more important, than a single lavage lymphocyte count in indicating progression towards chronicity and a likelihood of developing irreversible fibrosis. Even when the lavage lymphocyte count is combined with a ^{67}gallium scan, there is too great a discordance between these measurements to allow them to be used alone as indicators for treatment.

It has also been suggested that a neutrophil count of 10 per cent and greater and a positive ^{67}gallium scan in IPF distinguishes those with a tendency towards deterioration on steroids, while those with neutrophils of less than 10 per cent do better.[3] The arguments here are somewhat different. The tendency for IPF to progress is very much greater than in sarcoidosis and many series have now shown that about 50 per cent of patients die within four years of first presentation. Moreover, there is now considerable indirect information that a better response to steroids is achieved when the disease is at an earlier stage. Thus, a good case can be made for treating all cases (other clinical factors being acceptable) and not to rely on any arbitrary indicator, be it a physiologic, radiographic or lavage one. Further, while some cases with high neutrophils respond

to corticosteroids, those with a somewhat raised lymphocyte count respond particularly well and the neutrophil count in these is often relatively low. Thus, to depend on the neutrophil count alone is to deny the administration of steroids in perhaps the best (albeit minority) group of all.[4]

Lavage as a tool to monitor activity during treatment and to adjust drug dosage

Lavage in the context of adjusting therapy has, so far, been perhaps the most disappointing aspect of lavage as a clinical tool.

In sarcoidosis and hypersensitivity pneumonitis, lymphocyte counts often remain substantially raised when all other modalities of assessment have returned to normal. Few biopsies are available to establish the meaning of these results. That they do *not* necessarily reflect a consistently more sensitive index of activity is, however, suggested by the not infrequent finding of an improving radiograph and physiology in the presence of a normal lymphocyte count. Under these circumstances, the lymphocyte count appears to be relatively less sensitive.

In one study comparing cell counts in lavage samples before and during treatment of sarcoidosis, there was less discordance of results (i.e. one modality improving while another deteriorated) between the radiograph and gallium scan, the radiograph and angiotensin converting enzyme (ACE), and gallium scan and ACE, than between lavage lymphocytes and any of the other measurements.

Serial lavages in CFA have, with one exception, proved somewhat disappointing in that neutrophils and eosinophils frequently remain persistently raised in both deteriorating as well as stable steroid unresponsive cases. Thus, while deterioration seems to relate to raised neutrophils or eosinophils when patient *groups* are studied, this is not a consistent finding in individual patients, even when followed over several years (mean of four years in the particular study quoted) (personal observation). Of some interest is the observation that, in the small group of patients who responded well to steroids or cyclophosphamide, there was a trend of change towards normal in all types of inflammatory cell. Thus, it may be possible to use normalisation of counts as a marker for continuing remission of the underlying inflammatory process and, thus, allow for a greater reduction of doses of therapeutic agents. If these findings can be confirmed in even longer studies, the question of when drug therapy may be stopped in cases of fibrosing alveolitis may be answered.

Conclusion

The clinical value of bronchoalveolar lavage needs careful specification. In expert hands, and when special preparations or stains are used, it can be of considerable value diagnostically, both in terms of pathognomonic appearances and in suggesting a priority of diagnosis based on differential inflammatory cell counts.

In the author's view, lavage cell counts have not yet been proven to be reliable indicators upon which to make a decision to start treatment in either sarcoidosis or IPF. The frequent lack of concordance between the various modalities to assess "activity" and "progression of disease" make it unwise, at the present time, to rely on lavage lymphocyte counts for this purpose.

Likewise, serial lavage counts have not shown a close correlation with progression of disease when individual patients are studied and, in particular, patients with apparently stable disease may have continually raised neutrophil and eosinophil counts which persist over quite long periods of time. Counts do, however, tend to fall towards normal in those who have responded well and continued monitoring of a *normal* count may, perhaps, provide an additional method to aid monitoring of these patients while drug doses are being reduced. Thus, the value in this particular sub-group of patients, when an equally specific question is being asked, may be considerable.

Overall, alveolar lavage has provided an important new tool in attempting to understand disease. Its use by the clinician has been summarised and it can be extremely valuable, but changes observed in groups of patients cannot always be used to manage individuals. Thus, as indicated, the precise circumstances, in which it has value, have to be recognised. There is now considerable evidence that, over-reliance on particular types of cell count alone in individual patient management, is not justified. We must neither over- nor underestimate the clinical value of lavage, but use it sensibly in individual patients in the context of other information.

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REFERENCES

1. Ramirez RJ. Bronchopulmonary lavage; new techniques and observations. Dis Chest 1966; 50:581-8.
2. Crystal RG, Roberts WC, Hunninghake GW, Gadek JE, Fulmer JD, Line BR. Pulmonary Sarcoidosis: disease characterized and perpetuated by activated lung T lymphocytes. Ann Int Med 1981; 94:73-94.
3. Keogh BA, Line BR, Rust M, Hunninghake GW, Meier-Sydow J, Crystal RG. Clinical staging of patients with idiopathic pulmonary fibrosis. Am Rev Respir Dis 1981; 123 (Suppl):89.
4. Rudd RM, Haslam PL, Turner-Warwick M. Cryptogenic fibrosing alveolitis; relationships of pulmonary physiology and bronchoalveolar lavage to response to treatment and prognosis. Am Rev Respir Dis 1981; 124:1-8.

Enhancement of Cytostatic Activity of Macrophages Against MOPC-315 Tumour Cells by Combined Cyclophosphamide Administration and Immunisation with Glutaraldehyde-treated Tumour Cells*

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SUMMARY **Spleen cells of BALB/c mice injected with cyclophosphamide (CY:100 mg/kg i.p.), and one day later with glutaraldehyde-treated MOPC-315 tumour cells (GA-MOPC:25x10^6 cells/mouse i.p.), exhibited cytostatic activity *in vitro* against MOPC-315 tumour cells, when tested on the sixth day but not when tested on the 28th day after CY injection. Spleen cells from mice injected with CY alone or with GA-MOPC cells only were not cytostatic. The cytostatic activity (decrease in thymidine incorporation by tumour cells) was detected in unfractionated spleen cell suspensions, in spleen cell suspensions depleted of T cells, in the glasswool-adherent macrophage-enriched population but not in the nylon wool nonadherent T-enriched population. The cytostatic activity of the macrophage-enriched population was not affected by the addition of T cells. The T-enriched population was devoid of cytostatic activity even when supplemented with various amounts of macrophages. Most mice injected with CY and GA-MOPC cells were resistant to inoculation with a tumourigenic dose of MOPC-315 tumour cells.**

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It has been reported that cyclophosphamide (CY) protentiates cell-mediated immune responses,[1] such as delayed-type hypersensitivity,[2] contact sensitivity,[3] auto-immune response[4] and antitumour immune response.[5] Moreover, the eradication of established MOPC-315 tumours was assumed to be due to cooperation between the CY effect and the host's antitumour immunity.[6] It was also shown that the combination of CY with immunotherapy was more effective than of either one alone against tumours.[7-10] Potentiation of cell-mediated immunological response by CY was related to its selective effect on suppressor T cells[5,11-13] or to the selective depletion of B cells.[14] We have shown[15] that immunisation with syngeneic MOPC-315 tumour cells treated with glutaraldehyde (GA-MOPC cells) afforded partial protection against inoculation with a tumourigenic dose of MOPC-315 cells. The aim of the present work was to determine whether administration of CY would increase the immune response induced by the injection of GA-MOPC cells as expressed by the generation of cytostatic activity of spleen cells *in vitro* against MOPC-315 tumour cells.

MATERIALS AND METHODS

Mice and tumour

BALB/c male mice between 8 and 12 weeks of age, selected from a breeding colony of the Hebrew University at Jerusalem, were used in this study. The MOPC-315 myeloma cell line was derived from a plasma tumour originally induced in BALB/c mice by the intraperitoneal injection of mineral oil.[16] An *in vitro* line of MOPC-315 tumour cells, adapted to growth in culture,[17] was maintained by serial passages in RPMI 1640 medium

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(Grand Island Biological Company, Grand Island, NY, USA) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mmole/ml L-glutamine and 10 per cent foetal calf serum (complete medium). The viability of tumour cells in cultures was approximately 90 per cent as shown by trypan blue dye (0.1 per cent) exclusion test. The *in vitro* grown line was used in all experiments.

GA treatment of tumour cells (GA-MOPC)

Tumour cells were washed three times in PBS, pH 7.2, and resuspended at a concentration of $2x10^7$ viable cells/ml. A volume of 0.2 ml of the cell suspension was mixed with 1.8 ml of 0.02 per cent GA (TAAB, England) solution in PBS and kept for 10 minutes at room temperature. The GA-treated cells were washed by three subsequent centrifugations at 100xg for 7 minutes at 4°C and resuspended in PBS to a concentration of $5x10^7$ cells/ml for injections.

In vivo treatments

Groups of mice were injected with a freshly prepared solution of 100 mg/kg CY in distilled water (Taro, Haifa, Israel, lyophilised powder), i.p. on day 0, and one day later were immunised with $25x10^6$ GA-MOPC cells/0.5 ml i.p. and sacrificed on the sixth day (if not stated otherwise) for harvesting of spleen cells. Groups of untreated mice, of mice injected with CY only or of mice receiving only GA-MOPC cells served as controls. Mice injected with CY and GA-MOPC tumour cells and with CY alone was also inoculated i.p. with a tumourigenic dose of $5x10^4$ viable MOPC-315 tumour cells eight days after CY administration. The mortality of tumour-bearing mice was recorded and compared with that of inoculated, untreated mice.

Cell preparations

Intact spleen cell suspensions, T-depleted spleen cell suspension, macrophage-enriched and T-enriched spleen cell fractions were used. Spleen cell suspensions were prepared in RPMI medium (without FCS) and finally resuspended in complete RPMI medium supplemented with $5x10^{-5}$ M 2-mercaptoethanol. T-cell depletion was achieved by incubating $1x10^7$ viable spleen cells/ml with an equal volume of a 1:5,000 dilution of anti-Thy 1,2 monoclonal antibody (Booth, England) and an equal volume of 1:20 fresh guinea pig serum as the source of complement. Preliminary tests showed that approximately 98 per cent of thymocytes were killed by this procedure. A T-enriched fraction was obtained by passage through a glass-wool column[18] and subsequent passage through a nylon-wool column.[19] The nylon-wool nonadherent fraction contained more that 98 per cent anti-Thy 1,2 sensitive cells as shown by cytotoxic assay with monoclonal anti-Thy 1, 2 antibody. The macrophage-enriched population was obtained by elution of cells adherent to the glasswool column with warm PBS containing 0.02 per cent EDTA.[18]

In vitro Cytostatic assay

The assay is based on the inhibition of DNA synthesis of MOPC-315 tumour cells by spleen cells *in vitro* and was performed as described.[20] A mixture of 0.05 ml of $1x10^5$ spleen cells and 0.05 ml of $1x10^3$ target tumour cells per well (E/T ratio of 100/1) in complete medium supplemented with $5x10^{-5}$ M 2-mercaptoethanol was incubated in 96-well flat-bottom tissue culture plates (Linbro, USA) at 37°C in a humidified atmosphere of 5 per cent CO_2 for 24 hours. A quantity of 0.5 μCi (^{3}H) thymidine [(^{3}H) dT, Nuclear Center, Negev, Israel] in 0.05 ml/well was then added and the incubation was continued for another 18 hours. The cultures were terminated by adsorption onto glass filter disc papers by the use of an automatic harvester and counted in a Packard beta counter. Each combination mixture was performed in 4-6 parallel samples. The incorporation of (^{3}H) dT within MOPC-315 cells was calculated by substracting the background value of spleen cells alone from the value of total incorporation in mixed cultures of spleen cells and tumour cells. Parallel cultures containing mitomycin-treated tumour cells were also included in order to evaluate the possible stimulation of DNA synthesis in effector cells by tumour antigens. The rate of (^{3}H) dT incorporation did not differ appreciably between effector cells alone and effector cells mixed with mitomycin-treated tumour cells. For comparison, the rate of (^{3}H) dT incorporation was also determined in tumour cells incubated without the addition of Spleen cells.

Statistical analysis

The significance of cytostatic effect was evaluated by the Student's t test. Differences were considered significant when P was less than 0.05.

RESULTS

Antitumour cytostatic activity in intact spleen-cell populations

The cytostatic activity was evaluated in spleen cells of untreated mice, mice injected with CY and GA-MOPC cells and mice injected either with CY or with GA-MOPC cells. As shown in Figure 1, the intact spleen cell population taken on the sixth day from mice injected with CY 100 mg/kg (day 0) and $25x10^6$ GA-MOPC cells (day 1) exhibited cytostatic activity *in vitro* towards MOPC-315 tumour cells whereas spleen cells of normal mice, of mice injected either with CY or with GA-MOPC cells, were not cytostatic. Usually no significant difference was found in the rate of (^{3}H) dT incorporation between tumour cells incubated with spleen cells from normal mice and tumour cells incubated without the addition of spleen cells (Figs. 1,2,3).

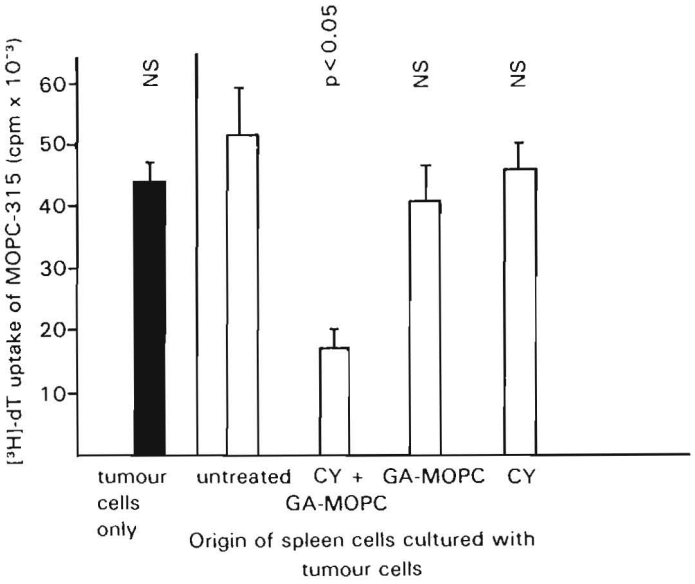

Fig. 1 Cytostatic activity of intact spleen cell populations towards MOPC-315 tumour cells following injection of cyclophosphamide (CY) and immunisation with glutaraldehyde treated MOPC-315 tumour cells (GA-MOPC), as expressed by the rate of [^{3}H] dT uptake. Tumour cells alone (■); tumour cells mixed with spleen-cell suspensions (□); CY (100 mg/kg) was given i.p. on day O; GA-MOPC (25 X 10^6/mouse) were injected i.p. on day 1; spleen cells were harvested on day 6 for evaluation of cytostatic activity; E/T - effector/target cell ratio of 100/1; P values were related to the mixture of tumour cells with spleen cells of the untreated mice; bars indicate mean ± SE of 4-6 paralled samples.

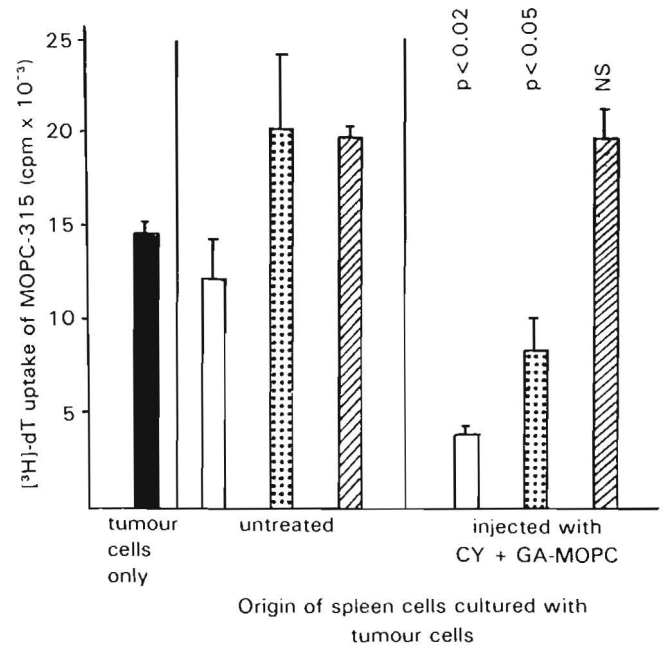

Fig. 2 Cytostatic activity of spleen cell populations towards MOPC-315 tumour cells following injection of CY and immunisation with GA-MOPC. Tumour cells alone (■); tumour cells were cultured with: intact spleen cell suspension (□); spleen cell suspension depleted of T cells (▦); and T-enriched nylon-wool nonadherent fraction of spleen cells (▨). See footnotes to Figure 1 for details.

Antitumour cytostatic activity in spleen-cell fractions

In view of the results showing cytostatic activity of spleen cells from mice injected with CY and GA-MOPC cells, attempts were made to define the spleen cell population responsible for this activity. The cytostatic activity towards MOPC-315 tumour cells was detected in a spleen cell population depleted of T cells but not in a nylon-wool nonadherent T-enriched population (Fig. 2). As shown in Figure 3, cytostatic activity was detected in the macrophage-enriched glass-wool adherent fraction (mostly macrophages) from CY+ GA-MOPC-treated mice but not in the same fraction obtained from normal untreated animals. The addition of various amounts of the macrophage fraction to a T-enriched population did not render the T fraction cytostatic. Thus, slight cytostatic activity, although not significant, was observed with the mixture of macrophages + T cells only when the quantity of macrophages added was $4x10^4$ cells out of a total of $1x10^5$ effector cells (Fig. 3, 1/2.5 ratio columm). When the quantity of macrophages in the mixture of M+T cells was less than $4x10^4$ no cytostatic effect was observed and the rate of thymidine incorporation was similar to that observed with the effector cell population containing only T cells (Fig. 3). The possibility that the T-enriched population contained cells suppressing the cytostatic activity of the macrophage-enriched fraction was examined by adding various quantities of cells from the T-enriched population to a cytostatic glasswool adherent fraction. As shown in Figure 4, a slight reduction in the cytostatic activity of macrophages against MOPC-315 tumour cells was observed in cultures supplemented with cells from a T-enriched population. However, the cytostatic activity in mixed cultures of T cells and macrophages was still significant. The persistence of cytostatic activity was examin-

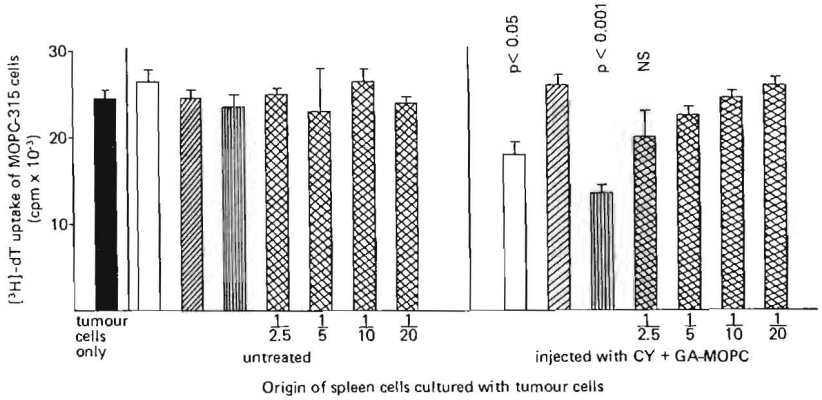

Fig. 3 Cytostatic activity towards MOPC-315 tumour cells in glass-wool adherent (macrophage-enriched) and T-enriched nylon-wool nonadherent spleen cell populations originated from mice injected with CY and GA-MOPC. Tumour cells alone (■); tumour cells were cultured with: intact spleen cell suspensions (□); T-enriched population (▨); macrophage-enriched population (▥); and T-enriched population completed to a total quantity of 1×10^5 cells/culture with the following amounts of macrophage-enriched population/culture (▩): 1/2.5, 1/5, 1/10 and 1/20. See footnotes to Figure 1 for details.

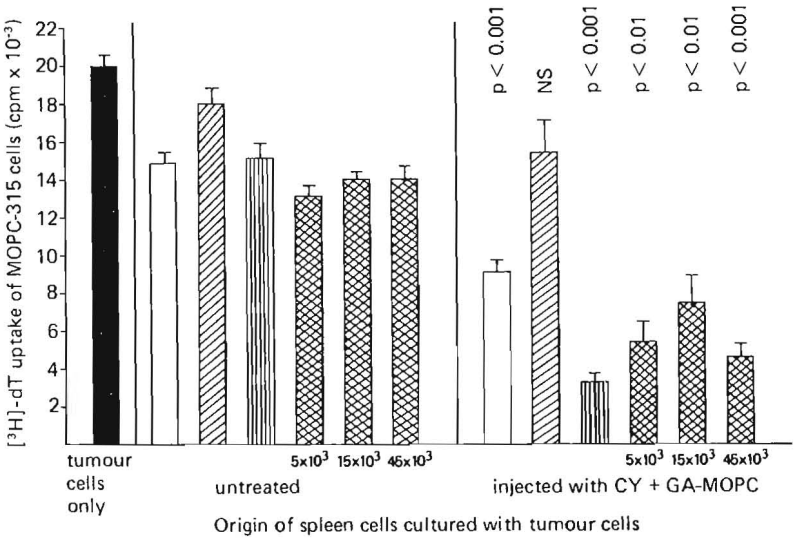

Fig. 4 Effect of addition of nylon-wool nonadherent (T-enriched) cells to macrophage enriched population on their cytostatic activity. Macrophage-enriched and T-enriched population originated form mice injected with CY and GA-MOPC. Tumour cells alone (■): tumour cells were cultured with: intact spleen cell suspension (□); T-enriched population (▨); macrophage-enriched population (▥); and macrophage-enriched population (1×10^5 cells/culture) supplemented with various amounts of T-enriched population (▩): 5×10^3 /culture, 1.5×10^4/culture and 4.5×10^4/culture. See footnotes to Figure 1 for details.

ed by evaluating this effect on the 28th day after the injection of CY (day 27 after immunisation with GA-MOPC cells), in comparison with day 7 after the CY injection (day 5 after immunisation with GA-MOPC cells). On the sixth day after the CY injection the cytostatic activity was again detected in the intact spleen cell population and in the glasswool adherent fraction but was not detected in spleen cells taken on the 28th day after the injection of CY (Fig. 5).

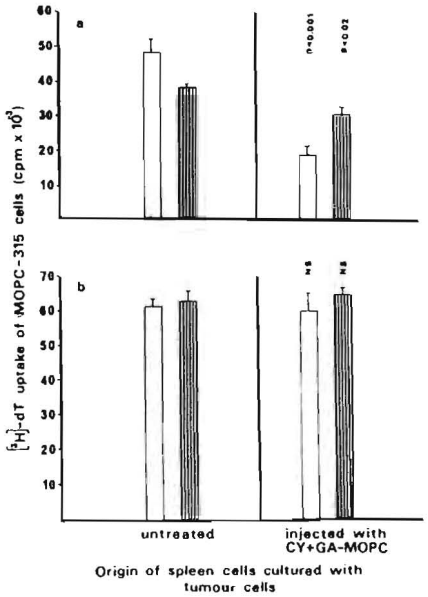

Fig. 5 Peristence of cytostatic activity in macrophage-enriched fraction of spleen cells from mice injected with CY and GA-MOPC. 5a - day 5 after CY injection; 5b - day 28 after CY injection. Tumour cells mixed with: intact spleen cell population (□); and macrophage-enriched population (▥). See footnotes to Figure 1 for details.

Tumour development in mice treated with CY and GA-MOPC cells

Mice injected with CY and GA-MOPC cells under conditions similar to those required for induction of cytostatic activity were inoculated with a tumourigenic dose of MOPC-315 tumour cells. As shown in Figure 6, most mice injected with CY and GA-MOPC cells, survived after the inoculation, whereas injection of CY alone had no effect on mortality. As shown previously,[15] a single injection of GA-MOPC did not induce a detectable level of protection against tumour inoculation.

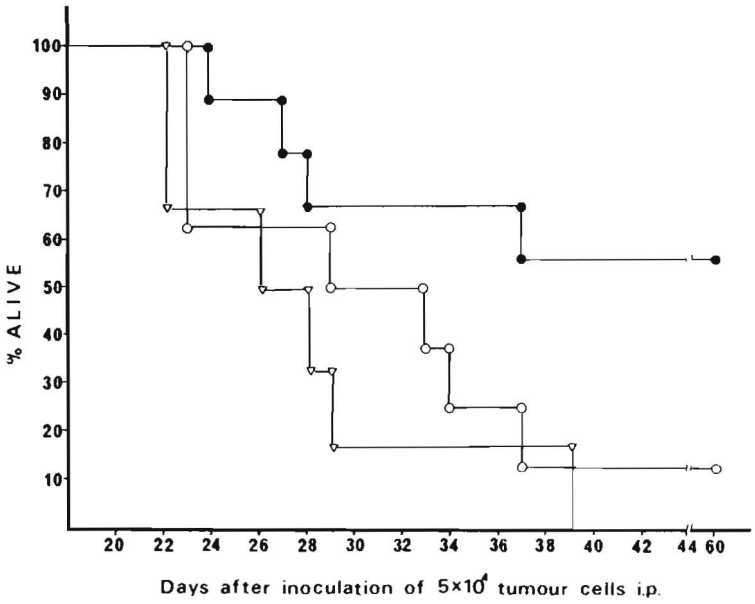

Fig. 6 Resistance of mice injected with CY and GA-MOPC to inoculation of MOPC-315 tumour cells. CY (100 mg/kg) injected in 0.5 ml i.p. on day 0. GA-MOPC cells (25×10^6/injection) injected on day 1. Viable tumour cells (5×10^4) were inoculated i.p. on day 8. (○—○) normal mice (8 mice per group); (▽—▽) CY injection mice (8 mice per group); (●—●) CY and GA-MOPC injected mice (9 mice per group).

DISCUSSION

The present experiments show that combined administration of cyclophosphamide and glutaraldehyde-treated syngeneic MOPC-315 tumour cells generated cytostatic activity against MOPC-315 tumour cells *in vitro*. On the other hand, injection of CY alone or of GA-MOPC cells only, did not induce cytostatic activity. It seems, therefore, that both CY and GA-MOPC injections are required for induction of cytostatic activity against tumour cells.

Selective effects of CY on suppressor T cells[5,11-13] and on B cells[14] were reported. It seems that under our conditions, the combined administration of CY and GA-MOPC cells resulted in increased macrophage activity. Thus, the cytostatic activity against MOPC-315 tumour cells was expressed by the macrophage-enriched fraction of spleen cells and was not detected in the T-enriched fraction. Moreover, the depletion of T cells from the intact spleen cell population did not affect its cytostatic activity against tumour cells. The lack of cytostatic activity in the T-enriched fraction is probably not due to dependence on macrophages for expression of cytostatic effect, because the addition of macrophages to the T-enriched fraction did not render this population cytostatic. The addition of T-cell fraction to macrophage-enriched fraction reduced slightly the cytostatic activity of macrophages against tumour cells. However, in view of the slight reduction in cytostatic activity, it does not seem possible to conclude whether the T-cell fraction contains a population suppressing the cytostatic effect. Although the full kinetics of the development and persistence of cytostatic activity has not been yet performed, it seems that this activity is transient as shown by its absence in spleen cells taken on the 28th day after CY administration.

Injection of CY may cause changes in the relative proportions of the different types of cells within the spleens, such as selective elimination of suppressor cells.[5,11-13] However, it seems unlikely that the induction of the cytostatic effect against MOPC-315 tumour cells by injection of CY and GA-MOPC cells is due exclusively to such changes because spleen cells taken from mice injected with CY alone were not cytostatic. Another possibility is that the thymidine incorporation assay in tumour cells is subjected to many errors when carried out in the presence of macrophages. This possibility seems also unlikely because the addition of unfractionated spleen cells or macrophage-enriched fraction of spleens from normal mice had no effect on the rate of thymidine incorporation in tumour cells.

It was reported[21] that cytotoxic activity against MOPC-315 target cells can be generated *in vitro* in cultures of spleen cells and that this activity is expressed by cytotoxic T cells. (For a review, see footnote[22]). It seems that the cytostatic activity reported here is different from the cytotoxic activity generated *in vitro* because in our hands, the T-enriched fraction was not cytostatic.

It was of interest to find out whether induction of cytostatic activity against tumour cells *in vitro* by CY and GA-MOPC administration has any bearing on the development of ascitic tumours of MOPC-315. We found in this respect, that most mice injected with CY and GA-MOPC cells survived after inoculation with an otherwise tumourigenic, lethal dose of MOPC-315 tumour cells, whereas injection of CY alone had no effect on the mortality rate after inoculation. We have also shown[15] that a single injection of MOPC-315 cells treated with 0.02 per cent GA did not confer protection against inoculation with a tumourigenic dose.

The mechanism of induction of cytostatic activity of macrophages against tumour cells by combined CY and GA-MOPC administration is not yet clear. A likely possibility[23] is that macrophages are activated

by soluble products released from T cells stimulated, in our case, by GA-MOPC antigen entity and that this stimulation is facilitated by CY-induced elimination of suppressor T cells.

ACKNOWLEDGEMENTS

We are grateful to Mrs. A. Jackman for excellent technical assistance, and to Mrs. Z. Gal for drawing the figures. This work was supported by grants received from the Moise and Frida Eskenasy Institute for Cancer Research, Tel-Aviv University, the Schreiber Fund, Tel-Aviv University, Israel, and the Israel Cancer Association by means of the Kessler Fund.

REFERENCES

1. Turk JL, Parker D. Effect of cyclophosphamide on immunological control mechanisms. Immunol Rev 1982; 65:99-113.
2. Askenase PW, Hayden BJ, Gershon RK. Augmentation of delayed-type hypersensitivity by doses of cyclophosphamide which do not affect antibody responses. J Exp Med 1975; 141:697-702.
3. Sy MS, Miller SD, Claman HN. Immune suppression with supraoptimal doses of antigen in contact sensitivity. I. Demonstration of suppressor cells and their sensitivity to cyclophosphamide. J Immunol 1977; 119:240-4.
4. L'Age-Stehr J, Diamantstein T. Studies on induction and control of cell-mediated autoimmunity. I. Induction of "autoreactive" T lymphocytes in mice by cyclophosphamide. Eur J Immunol 1978; 8:620-8.
5. Glaser M. Augmentation of specific immune response against syngeneic SV40-induced sarcoma in mice by depletion of suppressor T cells with cyclophosphamide. Cell Immunology 1979; 48:339-45.
6. Hengst JCD, Mokyr MB, Dray S. Cooperation between cyclophosphamide tumoricidal activity and host antitumor immunity in the cure of mice bearing large MOPC-315 tumors. Cancer Res 1981; 41:2163-7.
7. Chassoux DM, Gotch FM, Maclennan ICM. Analysis of synergy between cyclophosphamide therapy and immunity against a mouse tumor. Br J Cancer 1978; 38: 211-8.
8. Greenberg PD, Cheever MA, Fefer P. Eradication of disseminated murine leukemia by chemoimmunotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt-1 2 lymphocytes. J Exp Med 1981; 154:952-63.
9. Boyer CM, Kreider JW, Bartlett GL. Regulation of the expression of adoptive tumor rejection immunity by recipient cyclophosphamide sensitive cells. Cancer Res 1982; 4:2211-5.
10. Eberlein TJ, Rosenstein M, Spiess P, Wesley R, Rosenberg SA. Adoptive chemoimmunotherapy of a syngeneic murine lymphoma with long-term lymphoid cell lines expanded in T-cell growth factor. Cancer Immunol Immunother 1982; 13:5-13.
11. Gill HK, Liew FY. Regulation of delayed-type hypersensitivity. III. Effect of cyclophosphamide on the suppressor cells for delayed-type hypersensitivity to sheep erythrocytes in mice. Eur J Immunol 1978; 8:172-6.
12. Diamantstein T, Klos M, Hann H, Kaufmann SHE. Direct *in vitro* evidence for different susceptibilites to 4-hydroxycyclophosphamide of antigen primed T cell regulating humoral and cell mediated immune responses to sheep erythrocytes: A possible explanation for the inverse action of cyclophosphamide on humoral and cell-mediated immune responses. J Immunol 1981; 126:1717-9.
13. North RJ. Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. J Exp Med 1982; 155:1063-74.
14. Turk JL, Poulter LW. Effects of cyclophosphamide on lymphoid tissues labelled with 5-iodo-2-deoxyuridine- ^{125}I and ^{51}Cr. Int Arch Allergy 1972; 43:620-9.
15. Ben-Efraim S, Ophir R, Relyveld EM. Tumour immunoprophylaxis in mice using glutaraldehyde-treated syngeneic myeloma cells. Br J Cancer 1981; 43:554-7.
16. Eisen HN, Simms DS, Potter M. Mouse myeloma proteins with anti hapten antibody activity – the protein produced by plasma cell tumor MOPC-315. Biochemistry 1968; 7:4126-34.
17. Yaniv A, Gazit A, Dvir M, Guthmann D, Eylan E. Adaptation of murine MOPC-315 myeloma cells in growth *in vitro* and further characterization of their C-type viruses. Eur J Cancer 1978; 14:771-9.
18. Lowy I, Bussard AE. A simple method for the removal of antibody forming cells from whole spleen cell population. J Immunol Methods 1974; 5:107-10.
19. Julius MH, Simpson E, Herzenberg LA. A rapid method for the isolation of functional thymus derived murine lymphocytes. Eur J Immunol 1973; 3:645-9.
20. Flood PM, Phillips C, Taupier MA, Schreiberg H. Regulation of myeloma growth *in vitro* by idiotype-specific T lymphocytes. J Immunol 1980; 124:424-30.
21. Mokyr MB, Braun DP, Usher D, Reiter H, Dray S. The development of *in vitro* and *in vivo* anti-tumour cytotoxicity in non-cytotoxic, MOPC-315-tumour-bearer, spleen cells "educated" *in vitro* with MOPC-315 tumour cells. Cancer Immunol Immunotherapy 1978; 4:143-50.
22. Wagner H, Rollinghoff M, Nossal IGV. T-cell mediated immune responses induced *in vitro*: A probe for allograft and tumour immunity. Transplant Rev 1973; 17:3-36.
23. Meltzer MS. Tumour cytotoxicity by lymphokine-activated macrophages: Development of macrophage tumoricidal activity requires a sequence of reactions. In: Pick E, ed, Lymphokines. New York: Academic Press 1981; 3:319-43.

Survey of Immunoglobulin Levels in Atopic Families*

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SUMMARY **One hundred and ninety-six members (including both those affected by atopy and those not affected by it) of 39 atopic families, were assessed for immunoglobulin levels. There were no gross abnormalities in immunoglobulin G and M levels. Immunoglobulin E levels were high in 72 per cent of the whole population under study, especially in the patients affected by clinical atopy. Immunolobulin A levels were below normal mean values for age in 73 per cent of all family members regardless of their clinical picture (affected by atopy or healthy). The relationship of IgA levels and age were assessed and compared for members of atopic families and the non-atopic healthy control population. Control immunoglobulin levels from non-atopic individuals were obtained both from the literature and from the local population.**

ASIAN PACIFIC J ALLERG IMMUN 1984; 2 : 181-185.

Atopy is an inherited condition, as has been known for centuries; the mode of inheritance is believed to be polygenic.[1] The role played by environmental factors in the clinical expression of this inherited disease is emphasised by all authors;[2] however, many family studies have provided evidence of high heritability of IgE levels.[3] Measurements of the levels of other immunoglobulins –A, G and M– in atopic diseases show no gross abnormalities, although IgA deficiencies (transient or permanent) have been found in association with high IgE levels and clinical atopy.[4]

In the study presented here, we refer to immunoglobulin levels in a group of atopic families, all family members of which were investigated. The genetic pattern of IgE levels in these families was reported earlier.[5] In this paper we mainly study immunoglobulin A, G and M levels.

PATIENTS AND METHODS

One hundred and ninety-six individuals (parents and children) belonging to 39 atopic families were investigated. Ninety of them were clinically healthy and 106 affected by clinical atopy: asthma and eczema in nine of them, asthma and rhinitis in 16, asthma (only) in 39 and hay fever in 42 (Table 1). The clinical diagnosis was based on family history, case history, symptoms, physical examination, positive prick tests, high eosinophil counts, high IgE levels and good response to anti-allergic treatments. The reagents for prick tests were purchased from Hollister-Stier (U.S.A.). The extracts were 1:20 w/v for pollens, 1:50 w/v for dust mites and 1:10 w/v for moulds, in a solution of 50% glycerine as preservative, 0.5% sodium chloride and 0.27% sodium bicarbonate. Any wheal reaction 5 mm larger than the control was considered positive.

Immunoglobulin E levels were determined by Phadebas Prist technique and immunoglobulin A, G and M levels by immunodiffusion on Meloy plates. As mean normal values (N) and standard deviations (S.D.) for IgE, we used those given by Kjellmann[6] for individuals up to 14 years of age and those given by Barbee *et al* for individuals over 14 years[7] of age.

As mean normal values (N) and standard deviations (SD) for IgA,

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Table 1 Clinical picture of studied population: 39 atopic families

Diagnosis		No. of subjects
Healthy		90
Atopic	Asthma and eczema	9
	Asthma and rhinitis	16
	Asthma (only)	39
	Hay fever	42
	Total	106
Over all		196

G and M, we used those given by Stites.[8] A group of 177 healthy non-atopic, age-matched individuals who had no family history of atopy was assessed in our laboratory for immunoglobulin levels. The values obtained validated the international A, G and M normal values used for reference.

Statistical analysis

Log transformation was applied to all immunoglobulin levels obtained. Regression analysis was used to compare IgA values of healthy and atopic individuals in different age groups. A student T-test was used to assess the significance of differences between healthy and atopic individuals within the adult population.

RESULTS

The levels of IgG and IgM were evenly distributed around the mean normal values. IgE levels (histogram, Fig. 1) were above mean normal values (N) in 142 (72%) of the cases. Thirty-one of them (16%) were between N and N + 1 S.D., 27 (14%) between N + 1 S.D. and N + 2 S.D., and in 84 (42%) of the cases IgE values were above the N + 2 S.D. Elevated IgE levels appear in those family members who are affected by atopy, especially at a young age.

There is an overlap in the distribution of IgA levels (histogram, Fig. 2) of healthy and affected by atopy family members over all ages. In 144 (73%) of the cases IgA levels were below the mean normal (N) values for age. Fifty-six (28%) of them were between the N and N -1S.D., 71 (36%) between the N -1 S.D. and N -2 S.D. and in 17 (9%) of the cases IgA levels were below N -2 S.D. IgA values were particularly lower than normally expected in the age group between 11 and 30 years. Low IgA levels do not appear associated with the clinical status (healthy or affected by atopy).

Plots of standard IgA levels by age of the non-atopic population up to 20 years (Fig. 3) showed a steady increase of values with age, especially below the age of 10. Among members of atopic families (Figs. 4 and 5) there was a "knee effect" at the age of 10; a broken regression was fitted to this data. The regression line up to 10 years is $\log \text{IgA} = \underset{(26.3)}{3.723} + \underset{(4.7)}{0.123} \times \text{age}$ and above 10 years is $\log \text{IgA} = \underset{(17.0)}{5.033}$. All three coefficients were significantly non-zero (P value < 0.001) with the T-statistical values shown in parenthesis. IgA levels of members of atopic families over 20 years of age (Table 2) were significantly lower (P value < 0.001) than those of the control population (normal, non atopic individuals, not belonging to atopic families).

IgA levels of parents and children were compared within each one of the studied families (Table 3). The important results of this comparison are that in two families in whom IgA levels of both parents were over N -1 S.D.; the levels of three of their children were under N -2 S.D.; of five, between N -1 S.D. and N -2 S.D.; and of 12, over N -1 S.D. In eight families the IgA level of one parent was over N -1 S.D. and of the other under N -2 S.D.; IgA levels of three of their children were under N -2 S.D.; of 16 of them between N -1 S.D. and N -2 S.D.; and of 10, over N -1 S.D. In two families IgA levels of both parents were under N -2

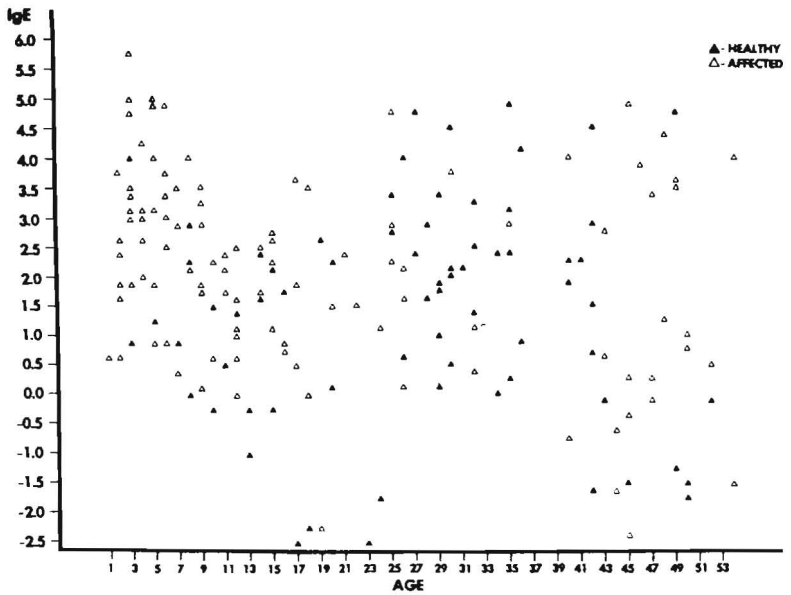

Fig. 1 IgE levels of whole population studied, standardised by age.

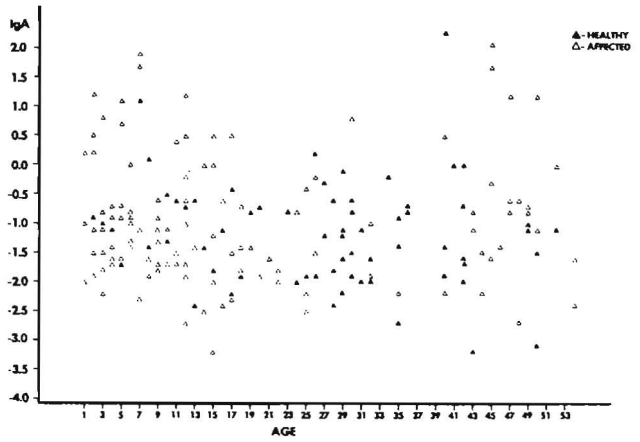

Fig. 2 Same for IgA levels.

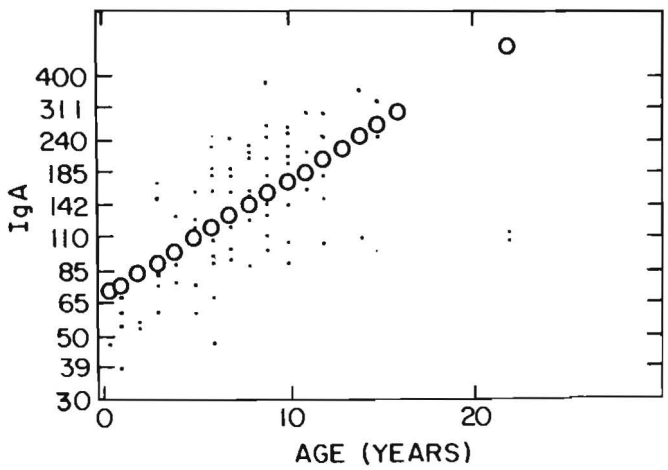

Fig. 3 Regression line of IgA levels (on log scale) of non-atopic control population up to 20 years of age (on background of individual values).

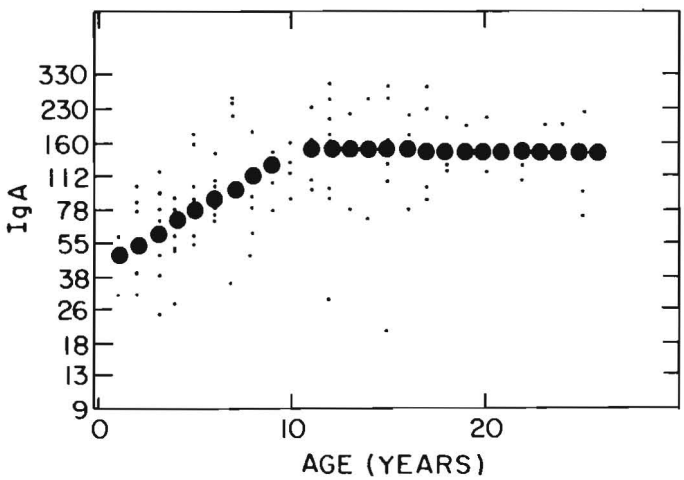

Fig. 4 Regression line of IgA values (on log scale) of members of atopic families up to 20 years of age (on background of individual values).

S.D.; IgA levels of two of their children were under N -2 S.D. and of two over N -1S.D.

DISCUSSION

Immunoglobulin E is now well established as the leading immunoglobulin for the diagnosis of an atopic background. However, abnormal values of other immunoglobulin classes have been found in cases of atopy.[9] Specifically, IgA levels are often low or even absent when atopy and high IgE levels are present.[10,12-14] Conley *et al*[15] described 11 IgA deficient patients with symptoms of autoimmune or atopic disease. Their IgA-bearing lymphocytes bore only small amounts of IgA in patchy distribution, similar to IgA-B cells from the newborn. They found that IgA-B cells from IgA-deficient patients were of an immature phenotype. Genetic factors in selective IgA deficiencies are mentioned by Amman[16] and reported by others.[17,18] Sloper *et al*[19] described low counts of IgA-producing plasma cells in the jejunal biopsies of children with atopic first-degree relatives.

Our results show an overall decrease in IgA levels over the population of atopic families studied with no difference between clinically healthy and atopic individuals. This decrease is not dramatic, and we certainly cannot consider it a classic deficiency. It is merely, as Soothill calls it,[20] "a low function within or near the normal range."

As shown in Figure 6, in members of atopic families there is a trend towards slow maturation of IgA levels which are less than N -1 S.D. until the age of 20 years. As can be seen from Table 2, in families with two normal IgA parents, 60 per cent of children have values that are normal and only 40 per cent have values less than N -1 S.D., whereas in families in which only one parent has a normal IgA value and the other an IgA value of less than N -1 S.D., 35 per cent of the children have normal IgA values and 65 per cent

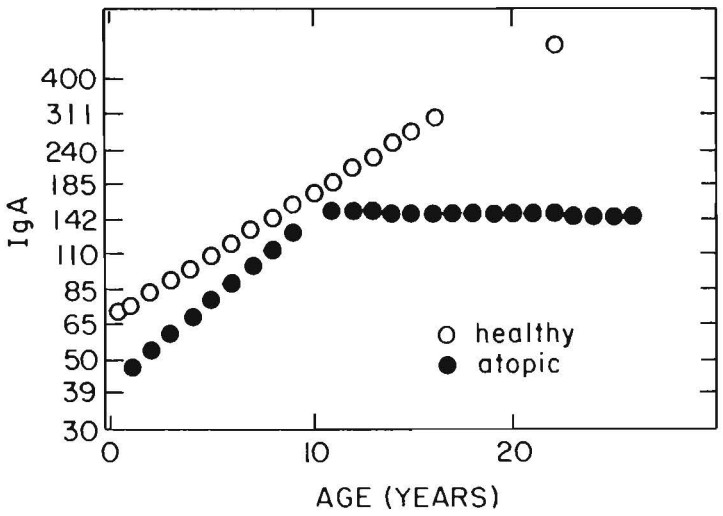

Fig. 5 Comparison between regression lines of IgA levels (on log scale) of non-atopic individuals and members of atopic families: note "knee effect" in the members of atopic families.

Table 2 Log IgA values for individuals over 20 years of age

Atopic families	No. of cases	Minimum	Maximum	Mean	S.D.
Members	78	3.21	6.07	5.07	.52
Non-atopic controls	68	4.700	5.91	5.53	.34

less than N -1 S.D. These findings were not indicative of dominant or codominant modes of inheritance.

In the 39 atopic families presented here, it seems that a low IgA levels is familial, possibly genetically determined by a polygenic mode of inheritance. Lower IgA levels were not predictive of clinical atopy within these families; however, because they appear as part of their familial "background", an inherited trait of low- and slow-maturing IgA globulin production might be an important factor in the pathogenesis of atopy.

REFERENCES

1. Soothill JF. The atopic child. In: Soothill JF, Hayward AR, Wood CBS, eds, Paediatric immunology. Oxford: Blackwell Sci Pub 1983; Chapter 15:251.
2. Marsh DG, Hsu SH, Hussain R, Meyers DA, Freidhoff MS, Bias WB. Genetics of the immune response to allergens. J Allergy Clin Immunol 1980; 65:322-32.
3. Buckley CE III. The genetics of allergy. In: Gupta S, Good RA, eds, Cellular, molecular and clinical aspects of allergic disorders. New York: Plenum Pub Corp, 1979; Chapter 7:274.

Table 3 Heritability of IgA levels within the 39 families studied

No. of families	IgA levels of parents	IgA levels of children			No. of children
		< N -2 S.D.	< N -1 S.D. and > N -2 S.D.	> N -1 S.D.	
5	Both >N – 1 S.D.	3	5	12	20
14	One >N – 1 S.D. N – 1 S.D. > One >N – 2 S.D.	4	21	14	39
8	One >N – 1 S.D. One <N – 2 S.D.	3	16	10	29
3	N – 1 S.D. >One >N – 2 S.D. One <N – 2 S.D.	1	4	2	7
7	N – 1 S.D. > Both >N – 2 S.D.	2	12	8	22
2	Both <N – 2 S.D.	2		2	4
(39)		(15)	(58)	(48)	(121)

N = mean normal value for age

Figures in parenthesis depict total numbers

4. Ostergaard PA. Clinical and immunological features of transient IgA deficiency in children. Clin Exp Immunol 1980; 40: 561-5.
5. Geller-Bernstein C, Weissglas L, Dun R, Tsur S, Kahane P, Lahav M. Immunoglobulin E and A levels in atopic and anaphylactic children. Ann Allergy 1980; 44:29-33.
6. Kjellmann N-IM, Johansson SGO, Roth A. Serum IgE levels in healthy children quantified by a sandwich technique (PRIST). Clin Allergy 1976; 6:51-4.
7. Barbee R, Halonen M, Lebowitz M, Burrows B. The distribution of IgE in a community population sample: correlation with age, sex and allergy skin test reactivity. J Allergy Clin Immunol 1981; 86: 106-11.
8. Stites DP. Clinical labo-atory methods for detection of antigens and antibodies. In: Fudenberg HH, Stites DP, Caldwell JL, Wells JV, eds, Basic and clinical immunology. Los Altos, Calif: Lange Med Pub, 1978; Chapter 27: 341.
9. Lorraine JB, Thong YH, Turner TW. The immunological status of children with atopic dermatitis. Acta Pediat Scand 1981; 70:551-5.
10. Burgio R, Duse M, Monafo V, Ascione A, Nespoli L. Selective IgA deficiency: clinical and immunological evaluation of 50 pediatric patients. Eur J Pediat 1980; 133: 101-4.
11. Kaufman HS, Hobbs JR. Immunoglobulin deficiencies in atopic population. Lancet 1970; 2:1061-3.
12. Herrod HG, Miller MM, Crawford LV, Lieberman PL, Holbert JM. Abnormal immunologic measurements in asthmatic children and adults. Ann Allergy 1981; 47:267-72.
13. Taylor B, Norman AP, Orgel HA, Turner MW, Stokes CR, Soothill JF. Transient IgA deficiency and pathogenesis of infantile atopy. Lancet 1973; 2:111-3.
14. Stokes CR, Taylor B, Turner MW. Association of house-dust and grass-pollen allergies with specific IgA antibody deficiency. Lancet 1974; 2:485-7.
15. Conley ME, Cooper MD. Immature IgA B cells in Ig A-deficient patients. N Engl J Med 1981; 305:495-7.
16. Amman AJ, Hong R. Disorders of the IgA system. In: Stiehm ER, Fulginity VA, eds, Immunologic disorders in infants and children. Philadelphia: WB Saunders, 1980:264-5.
17. Perez-Jimenez F, Lopez PB, Tallo EP, Guzman JR, Molina JS, Pereperez JAJ. Selective IgA deficiency and the HLA-B8 antigen. Arch Intern Med 1981; 141:509-10.
18. Fisher SE, Smith WI Jr, Rabin BS, Tomasi TB Jr, Lester R, Van Thiel DH. Secretory component and serum immunoglobulin A deficiencies with intestinal autoantibody formation and autoimmune disease: a family study. J Ped Gastroenterol Nutr 1982; 1:35-42.
19. Sloper KS, Brook CGD, Kingston D, Pearson JR, Shiner M. Eczema and atopy in early childhood: low IgA plasma cell counts in the jejunal mucosa. Arch Dis Child 1981; 56:939-42.
20. Soothill JF. Some intrinsic and extrinsic factors predisposing to allergy. Proc Roy Soc Med 1976; 69:439-42.

Induction of Suppressor T Cells of Immunoglobulin Production by Alpha-foetoprotein in An *In Vitro* Human System*

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Junichi Yata, M.D.

SUMMARY **Human T cells treated with alpha-foetoprotein (AFP) showed a suppressor effect on the generation of immunoglobulin-producing cells from lymphocytes stimulated with pokeweed mitogen (PWM). This effect seemed to be mediated by soluble factor(s), since the culture supernatant of AFP-treated T cells showed the same suppression. The suppressor factor(s) blocked the differentiation of B cells helped by T cells or mitomycin C-treated T cells, but not the B cells helped by soluble helper factor(s) from T cells. Therefore, the AFP-induced suppressor T cells seemed to exert their effect via helper T cells, but not directly on B cells. The induction of suppressor T cells might be one of the immunosuppressive mechanisms of AFP.**

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It is well known that alpha-foetoprotein (AFP) inhibits in *in vitro* proliferation of human lymphocytes in response to mitogens or in mixed lymphocyte culture reaction.[1-3] Similarly, AFP has been shown to exert an immunosuppressive effect on antibody synthesis in mice.[4] With regard to this immunosuppressive mechanism, suppressor T cells have proven to be induced by AFP in experimental animals.[5] It has been reported that the newborn T cells of newborn humans or cord blood T cells suppress the pokeweed mitogen (PWM)-driven T-cell-dependent differentiation of B cells to plasma cells.[6,7] These facts have suggested that AFP also induces suppressor T cells in human lymphocytes.

This paper demonstrates that AFP induces human T-cell activity which suppresses immunoglobulin (Ig) production by lymphocytes stimulated with PWM and that the suppression is mediated by soluble factor(s) secreted from suppressor T cells acting upon helper T cells.

MATERIALS AND METHODS

Peripheral blood lymphocytes

Heparinised venous blood drawn from healthy adults was gently layered on Ficoll-Hypaque density solutions (s.g., 1.074) and centrifuged at 400xg for 30 minutes. The mononuclear cells were collected from the interface and washed three times with phosphate buffered saline solution (pH 7.4) (PBS).

Separation of non-T cells (B cell-rich fraction) and T cells

The mononuclear cells were mixed with neuraminidase-treated sheep erythrocytes in a ratio of about 1:100 in inactivated foetal calf serum (FCS) (Gibco), centrifuged at 200xg for 5 minutes to obtain tight cell contact. They were kept in ice water for one hour for the T cells to form rosettes with the sheep erythrocytes. The cells were then gently resuspended and applied to Ficoll-Hypaque density gradient. The cells that formed rosettes (T cells) were collected from the bottom; non-T cells (B-cell-rich fraction), from the interface. The contaminating erythrocytes were removed by lysing with tris-buffered 0.83% NH_4Cl.

The preparation of the human foetal α-foetoprotein

An enriched soluble preparation of human foetal α-foetoprotein was purchased from Calbiochem-Bering Co., Ltd. This solution was further fractioned by Sephadex-G 200 gel

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chromatography. The fractions with molecular weights ranging from 60,000 to 80,000 daltons were collected and concentrated by Amicon filtration. AFP was further purified from this fraction by immunoaffinity chromatography using rabbit anti-AFP antibody (Calbiochem-Bering Co., Ltd.) coupled to CNBr-activated Sepharose 4B. The concentration of AFP in this preparation was measured by immunoprecipitation.

Treatment of T cells with AFP

T cells ($5x10^5$) were incubated at 37°C with various concentrations of AFP (0.1, 1, 10, 20, 50, and 100 μg/ml) for various lengths of time ranging from 3 to 72 hours, in a humidified, 5% CO_2 incubator. The cells were then washed three times with PBS and resuspended in fresh culture medium (RPMI 1640 supplemented with 10% FCS (Gibco), 100 μg/ml gentamicin, and 100 units/ml penicillin-G).

Preparation of soluble suppressor factor(s)

T cells ($1x10^6$) in 1 ml of the medium containing various concentrations of AFP ranging from 10 to 100 μg/ml were cultured for 24 hours at 37°C in a humidified, 5% CO_2 incubator. After washing three times with PBS, they were then cultured in fresh medium for another four days before the cell-free supernatant was collected. The supernatant, after undergoing five-fold concentration by Amicon ultrafiltration, was used as a source of suppressor factor(s) in further experiments. The culture supernatants of untreated T cells were used as controls.

Preparation of soluble helper factor(s)

Non-T cells (B-cell-rich fraction) ($1x10^6$) in 1 ml of the medium were treated with 50 μg/ml mitomycin C (MMC) (Sigma) for 30 minutes at 37°C, in a humidified, 5% CO_2 incubator. A mixture of MMC-treated non-T cells ($5x10^5$) and allogenic T cells ($5x10^5$) in one millilitre of medium was cultured at 37°C for four days in a humidified, 5% CO_2 incubator. The cell-free supernatant was collected, concentrated five-fold using an Amicon filter, for use as a source of helper factor(s) in further experiments.

Assay for suppressor activity of T cells pretreated with AFP or of the culture supernatant from pretreated cells

The suppressor effect of T cells treated with AFP or of their culture supernatant (suppressor factor(s)) was assayed by the decrease of the immunoglobulin-producing cells (Ig-PCs) generated from the mixture of autologous non-T cells and T cells stimulated with PWM. T cells treated with AFP ($2x10^5$) or 0.2 ml of the five-fold concentrated culture supernatants from pretreated cells were added to the mixture of non-T cells ($2x10^5$) and T cells ($2x10^5$) and the cells were suspended in a culture tube (Falcon, 2054) in a total volume of 1 ml of medium containing 5 μg/ml of PWM. The cells were cultured at 37°C for seven days in a humidified, 5% CO_2 incubator. In some experiments, the T cells used to help the B cells were treated with 25 μg/ml of MMC at 37°C for 30 minutes to eliminate the effect of suppressor cell activity which is MMC-sensitive.[8] A count was made of the number of cells at the end of the culture. The cells were smeared on a glass slide, fixed with acetone for 10 minutes, stained with fluorescin-conjugated anti-human immunoglobulin antibody and observed under a UV-microscope. The percentage of cells showing cytoplasmic fluorescence (Ig-PCs) was then scored. The suppressor activity (i.e. the reduction in the number of Ig-PCs) was expressed in term of this number or as a ratio of this number in the presence of added T cells treated with AFP or their culture supernatants, relative to controls containing untreated T cells or their respective supernatants.

Effect of the suppressor factor(s) on the B cell differentiation exerted by the helper factor(s)

The helper factor(s) was added to non-T cells (B cell-rich fraction) ($4x10^5$) in a volume constituting 20 per cent of the entire culture medium and the cells were cultured in the presence of 5 μg/ml of PWM. These factors facilitated the B cell differentiation to Ig-PC, when tested by the aforementioned method. The suppressor factor (s) was added to this system as 20 per cent of the final culture volume and its effect was studied.

RESULTS

Suppressor activity of T cells treated with AFP

The T cells showed suppressor activity when they were treated with 50 μg/ml or more of AFP for longer than 12 hours (Fig. 1). The maximal suppressor activity was obtained by treatment with these concentrations for 24 hours or more.

Suppressor activity of the culture supernatant from T cells treated with AFP

The suppressor effect of the culture supernatant was demonstrated when T cells were treated with 50 μg/ml or more of AFP. The generation of Ig-PCs was suppressed by more than 50% (Fig. 2). The treatment with 20 μg/ml of AFP exerted some suppression but its effect was not sufficient.

Effect of the suppressor factor(s) on the differentiation of B cells co-cultured with T cells treated with mitomycin C

It is known that suppressor T cells are usually more sensitive to antimiotic drugs than helper T cells[9] and, therefore, most of the suppressor activity can be removed without affecting helper T cell function by using adequate doses of mitomycin C (MMC). The suppressor factor(s)

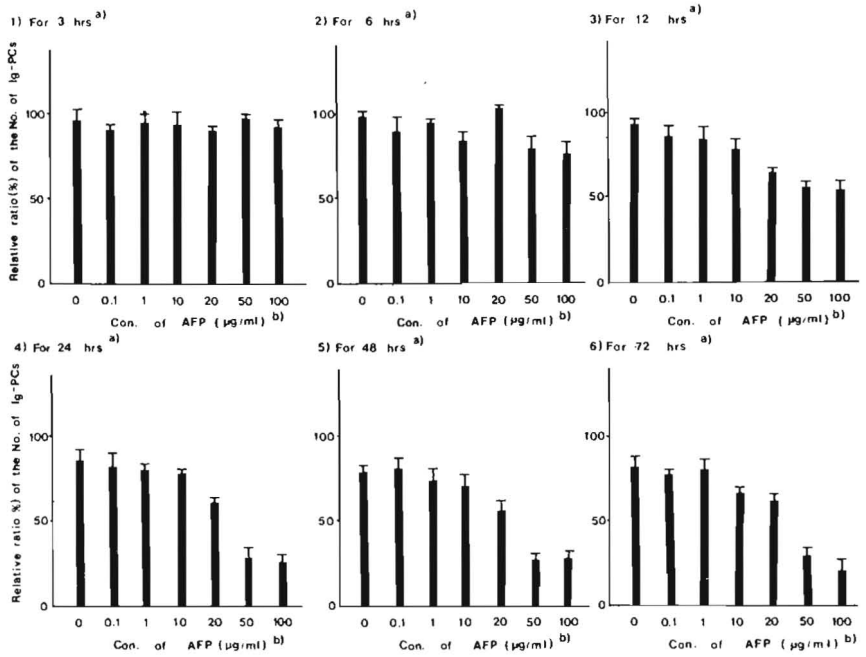

Fig. 1 Effect of AFP-treated T cells on the generation of immunoglobulin-producing cells (Ig-PCs) from the lymphocytes stimulated with PWM.
The percentages indicate the relative scores of the number of Ig-PCs from the combination of non-T cells and T cells added to T cells treated with AFP as compared with that of the combination of non-T cells and T cells alone.
a): Length of time for the treatment by AFP
b): The concentration of AFP used for the treatment of T cells.

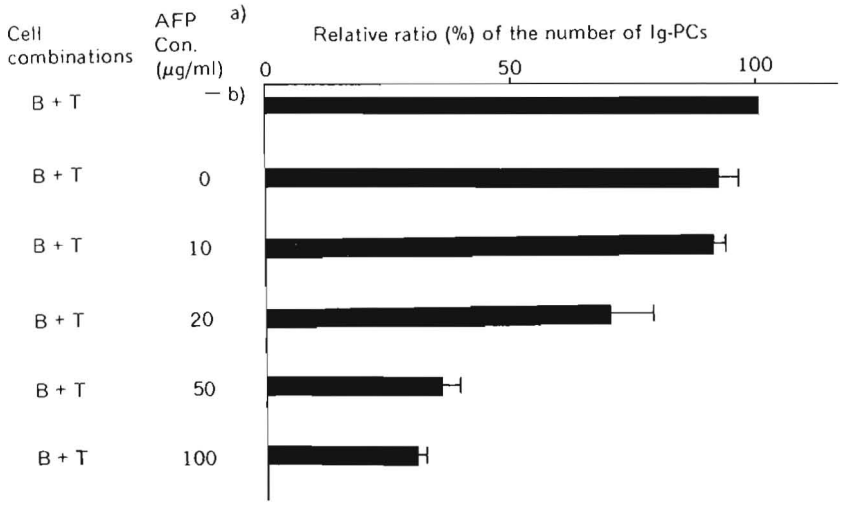

Fig. 2 Suppressor effect of the culture supernatant from T cells treated with AFP on the generation of Ig-PCs from the lymphocytes stimulated with PWM.
The percentages indicate the relative scores of the number of Ig-PCs from the combination of the non-T cells and T cells added to the supernatant as compared to that of the mixture of non-T cells and T cells alone.
B + T: Combination of non-T cells and T cells
a): The concentration of AFP used for the treatment of T cells from which the culture supernatant was obtained.
b): Control supernatant was added.

elicited their suppressor effect on B cell differentiation helped by MMC-treated T cells as well (Fig. 3). This indicates that the factor(s) exert their function, not by inducing MMC-sensitive suppressor T cells, but by directly working as a suppressor itself.

Effect of the suppressor factor(s) on B cell differentiation by helper factor(s)

The cell-free supernatant obtained from the mixed lymphocyte culture reaction facilitated the B cell differentiation on the Ig-PCs in response to PWM by functioning as helper factor(s). Suppressor factors from T cells treated with AFP did not inhibit the B cell differentiation helped by this factor(s) (Fig. 4). Therefore, the suppressor factors seemed to have no direct effect on B cells.

DISCUSSION

It has been shown that serum from pregnant women can suppress the mitotic response of lymphocytes.[10] Similarly, amniotic fluid inhibited the formation of antibody to sheep erythrocytes and AFP was found to be the causative agent for this suppression.[4,11] It was also reported that non-specific suppressor T cells induced by AFP seemed to be related to immunosuppression.[12] There were few reports with regard to the effect of AFP on the regulatory mechanisms of immunoglobulin production from peripheral blood lymphocytes in humans. T cells from cord blood in which AFP was present at a high concentration showed suppressor activity *in vitro*.[13,14]

This paper demonstrates that the T cells exerting suppressor activity in immunoglobulin formation are induced by concentrations of AFP physiologically present in the blood of newborn infants, and emphasises their role in the immunosuppressive mechanisms of AFP.

By studing various conditions, it was found that 50 μg/ml of AFP or

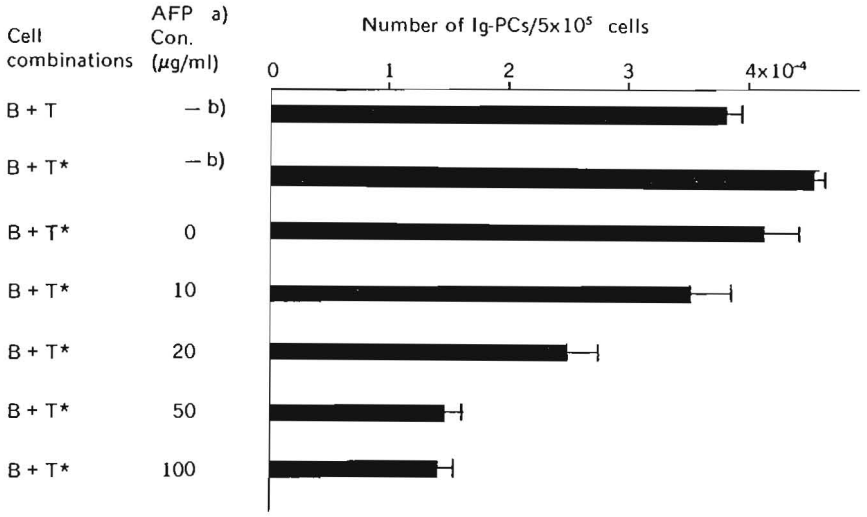

Fig. 3 Effect of the cell-free culture supernatant of AFP-treated T cells on the generation of Ig-PCs from non-T cells helped by T cells pretreated with mitomycin C (MMC).
B + T : The combination of non-T cells and T cells
B + T*: The combination of non-T cells and T cells treated with MMC.
a): The concentration of AFP used for treatment of T cells from which the culture supernatant was obtained.
b): Control supernatant was added.

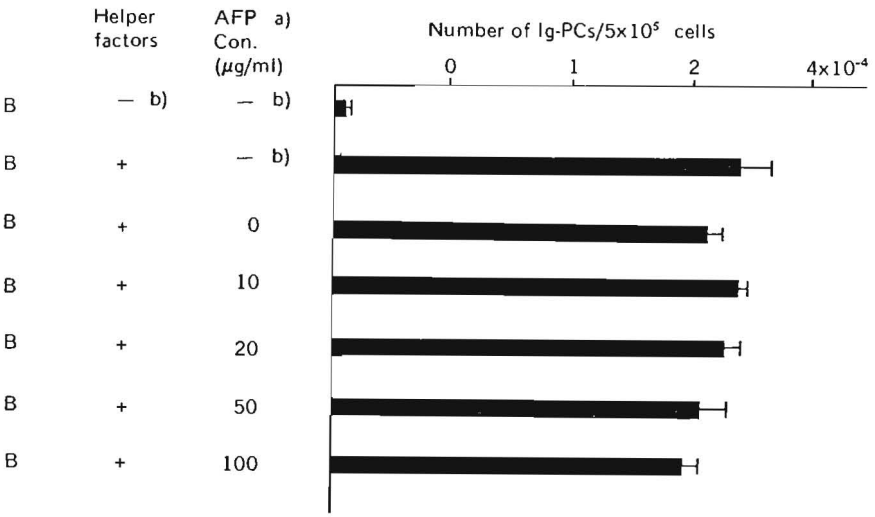

Fig. 4 Effect of the culture supernatant of AFP-treated T cells on B cell differentiation helped by soluble factor(s).
B : non-T cells (B-cell-rich fraction)
a): The concentration of AFP used for treatment of T cells from which the culture supernatant was obtained.
b): Control supernatant was added.

more, and a minimum of 12 hours of pre-culture were necessary to induce the suppressor T cells. The concentration of AFP which could induce suppressor T cells was approximately related to the mean concentration in the cord sera.[15] The cell-free supernatant from T cells pre-treated with AFP similarly suppressed the generation of Ig-PCs. Therefore, suppression by T cells treated with AFP seems to be mediated by soluble factor(s).

Our present data discloses that suppressor factor(s) from AFP-treated T cells suppressed B cell differentiation helped by MMC-treated T cells, but not that exerted by the helper factor(s) obtained from mixed lymphocyte culture reactions.[16] This suggests that the suppressor factor(s) inhibit helper T cells but have no direct effect on B cells. Other authors have reported that AFP does not exert a suppressive effect on B cells responding to thymus-independent antigens.[17] The factor(s) seemed to act as the suppressor(s) itself, and not through the induction of suppressor T cells, since it also suppressed the differentiation of B cells co-cultured with MMC-treated T cells, where the activation of most suppressor T cells was inhibited.[9]

These observations suggest that the immunosuppressive effect of AFP is due at least in part to the induction of suppressor T cells and that the activation of suppressor T cells observed in newborn infants might be caused by AFP which is present in high concentrations in the blood of foetuses and newborn in fants.

REFERENCES

1. Auer IO, Kress HG. Suppression of the primary cell-mediated immune response by human alpha 1-fetoprotein. Cell Immunol 1977;30:173-9.
2. Gupta S, Good RA. Alpha-fetoprotein and human lymphocyte subpopulations. J Immunol 1977;118:405-8.
3. Murgita RA, Goild E, Kontianen S, Wigzell H. Analysis of the immunosuppressive activity of human and mouse alpha-fetoprotein. Fed Proc 1977;36:1318.
4. Murgita RA, Tomasi TB. Suppression of the immune response by alpha-fetoprotein on the primary and secondary antibody response. J Exp Med 1975;141:269-86.
5. Murgita RA, Goild E, Kontianen S, Wigzell H. Alphafetoprotein induces suppressor T cells in vitro. Nature 1977;267:257-9.

6. Miyawaki T, Seki H, Kubo M, Taniguchi N. Suppressor activity of T lymphocytes from infants assessed by co-culture with unfractionated adult lymphocytes in the pokeweed mitogen system. J Immunol 1979; 123:1092-6.
7. Hayward AR, Lydyard PM. Suppression of B lymphocyte differentiation by newborn T lymphocytes with Fc receptor for IgM. Clin Exp Immunol 1978; 34: 374-8.
8. Hoffstein PE, Dixon FJ. Effect of irradiation and cyclophosphamide on anti-KLH antibody formation in mice. J Immunol 1974; 112:564-72.
9. Stevenson HC, Fauci AS. Activation of human B lymphocytes. XII differentiational effects of *in vitro* cyclophosphamide on human lymphocyte subpopulation involved in B-cell activation. Immunology 1980; 39:391-7.
10. Figuerdo MA, Palomino P, Ortiz F. Lymphocyte response to phytohaemagglutinin in the presence of serum from pregnant women; correlation with serum levels of alpha-fetoprotein. Clin Exp Immunol 1979; 37:140-4.
11. Ogra SS, Murigita RA, Tomasi TB. Immunosuppressive activity of mouse amniotic fluid. Immunol Commun 1974; 3:497-508.
12. Gershwin ME, Castles JJ, Ahmed A, Makishima R. The influence of alpha-fetoprotein on Moloney sarcomavirus oncogenesis: evidence for generation of antigen nonspecific suppressor T cells. J Immunol 1978; 121:2292-8.
13. Andersson U, Bird AG, Britton S, Palacios R. Humoral and cellular immunity in humans studied at the cell level from birth to two years of age. Immunol Rev. 1981; 57:1-38.
14. Hayward AR. Development of lymphocyte response and interactions in the human fetus and newborn. Immunol Rev 1981; 57:39-60.
15. Tuchida Y, Endo Y, Saito S, Kaneko M, Shiraki K, Ohmi K. Evalution of alpha-fetoprotein in early infancy. J Pediatric Surg 1978; 13:155-62.
16. Parker DC. Separable helper factors support B cell proliferation and maturation to Ig secretion. J Immunol 1982; 129: 469-74.
17. Murgita RA, Andersson LG, Sherman MS, Bennich H, Wigzell H. Effect of human alpha-fetoprotein on human B and T lymphocyte proliferation *in vitro*. Clin Exp Immunol 1978; 33:347-56.

Alpha-foetoprotein in Chronic Lymphocytic Leukaemia*

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Jaffa Nothman
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SUMMARY **Alpha-foetoprotein (AFP) antigen was detected by using the indirect fluorescent technique on lymphocytes from 12 patients with chronic lymphocytic leukaemia (CLL). The percentage of AFP antigen-positive cells varied from case to case and seemed to be highly increased on T-enriched lymphocytes from CLL patients. Preincubation of the patients' lymphocytes with anti-AFP antibody had an enhancing effect on the E-rosette binding capacity of the treated cells. This capacity was significantly increased by prolonged incubation of the treated lymphocytes with sheep red blood cells (SRBC) at 4°C. Preincubation of the patients' lymphocytes with anti-AFP was found to interfere with the T-cell Fc-surface receptor for IgG complexes, suggesting the possibility that the carcinofoetal antigen may be located on the cell membrane of this subpopulation of T-suppressor cells.**

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The presence of a membrane receptor for alpha foetoprotein (AFP) antigen on normal lymphocytes as well as its immunosuppressive effect on these cells[1-4] make especially interesting investigations on this antigen in lymphoproliferative disorders. Oncofoetal antigens were found in patents with Hodgkin's disease,[5,6] in cases of sarcoma,[7] and in circulating lymphocytes of patients with lymphomas.[8]

In view of some antigenic similarities between foetal and CLL lymphocytes,[9] the different distribution of the major membrane glycoproteins on the lymphocyte surface of normal and CLL T and B lymphocytes,[10] the relative immunological inertness and immunoincompetence of the circulating lymphocytes in CLL patients,[11,12] we found that it may be of interest to examine these cells for the possible presence of a membrane-bound oncofoetal antigen. The current preliminary study was initiated to investigate whether experimental evidence can be obtained on the presence of AFP antigen on lymphocytes from CLL patients. Based on the observations that AFP can suppress certain T-cell-dependent function,[2-4] the possible correlation of this antigen with the patients' T-lymphocyte markers was also considered.

MATERIALS AND METHODS

Patients

The study comprised 12 patients with CLL (Table 1). Their disease status, according to Ray's[13] clinical staging of CLL, was classified from 0 to III. Eight of the 12 patients were in a mild to moderate stage (0-I), and patients 4, 8, 9 and 12 (Table 1) in a more severe stage (II-III); three of them received anti-leukaemic treatment at the time of examination (Table 1). Seven healthy blood bank donors (male) served as controls.

Methods

The indirect fluorescent technique was used to obtain experimental evidence of AFP antigen on the patients' lymphocytes. To observe whether the antigen can be correlated with the patients' T-cell membrane, the modulating effect of anti-AFP antibody treatment in low concentration on T-cell-specific surface markers, such as spontaneous E-rosette formation with SRBCs and the T-lymphocyte Fc IgG receptor, characteristic for a subpopulation of T-suppressor cells,[14] was examined. For all tests, mononuclear cells were obtained from Hypaque-Ficoll sedimentation of peripheral heparinised blood.[15]

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Table 1 The effect of anti-AFP antibody treatment on membrane fluorescent staining and E-rosette formation of lymphocytes from CLL patients

Patients	Sex	Total WBC/μl	Lymphocytes %	Therapy	Fluorescent* %	E rosette % – 1 hour at 4°C		
						Untreated cells	Medium	Anti-AFP**
1	M	133,000	96	–	14	7	6	12
2	M	26,800	85.	–	15	9	15	20
3	M	32,000	91	–	20†	6	11	12
4	M	493,000	80	Leukeran	10	4	4	7
5	M	30,200	84	–	15	6	10	24
6	M	36,000	83	–	10	9	8	8
7	M	19,400	78	–	16	10	16	20
8	M	42,300	95	Meticorten	10	10	12	21
9	F	63,000	87	–	12†	6	8	21
10	F	47,700	86	–	19	14	13	11
11	F	14,100	60	–	18†	26	28	32
12	F	42,500	96	Meticorten	15	4	3	3

*Anti-AFP dilution 1:10

†The percentage of fluorescent staining cells in T-lymphocyte preparations was 30%, 38% and 35%, respectively.

**Anti-AFP 1:200, Student's paired "t" test for statistical evaluation. Preincubation with medium had no significant effect on the E-rosette formation. Preincubation with anti-AFP significantly enhanced the percentage of E rosettes ($P < 0.01$).

Indirect immunofluorescent studies

To demonstrate specificity of the immunofluorescence, the fluorescein-conjugated antibody (7S goat anti-rabbit IgG-Hyland) was absorbed overnight at 4°C on the respective normal or leukaemic lymphocytes. The remaining lymphocytes resuspended in RPMI 1640 supplemented with 5% inactivated normal pooled human serum were stored overnight at 37°C in 5% CO_2 humidified air, washed in phosphate buffered saline (pH = 7.3), counted, incubated for one hour with specific rabbit antihuman AFP antiserum at 37°C (Behring, 0.4 mg/ml antibody), washed and reincubated with the absorbed fluorescent anti-rabbit antiserum (concentration 1:8, 1:5). The thoroughly washed cells were examined for fluorescence under an IV F_1 epifluorescence condensor with an HBO 50W superpressure mercury lamp. For pictures, a dark-field condensor with translumination was added. For control, heat-inactivated absorbed normal rabbit serum and rabbit anti-human albumin (Behring) were used instead of anti-AFP antiserum. For anti-AFP blocking experiments, AFP human standard serum (Behring) was employed to demonstrate the specificity of immunofluorescence.

E rosettes with SRBCs

Patients' and controls' lymphocytes pretreated with anti-AFP antiserum (Behring, concentrations 1:10, 1:20, 1:200) for one hour at 37°C in a humidified atmosphere of 5% CO_2, washed, counted for viability (92-94%), were tested for their capacity to form E rosettes.[16] For each patient, untreated lymphocytes and lymphocytes treated in medium only were used for control. In a number of cases (patients and controls), the effect of a prolonged incubation time (24 hours at 4°C) for the E-rosette formation of anti-AFP treated and untreated cells was studied.

T lymphocytes with IgG receptors (Tγ cells)

In seven patients and six controls, the presence of the Fc receptor for IgG complexes was tested on enriched T lymphocytes. T lymphocytes were purified from non T cells by rosetting with SRBCs which were lysed from the lymphocytes using a hypotonic shock, a short exposure to 0.24% NaCl. The enriched T cells were incubated as previously described with anti-AFP antiserum, thoroughly washed and used for the determination of T cells by rosette formation with ox erythrocytes coated with the IgG fraction of rabbit anti-ox erythrocyte antibody.[14] Anti-ox red cell Ig antibody was raised in rabbits, purified over a Sephadex G-200 column and controlled for IgG specificity by immunoelectrophoresis.

RESULTS

Indirect immunofluorescence studies

The percentage of positive stained cells (Fig. 1) varied from case to case (Table 1), and could be observed at antibody dilutions of 1:10 and 1:20. No significant lymphocyte surface staining could be observed with inactivated, absorbed

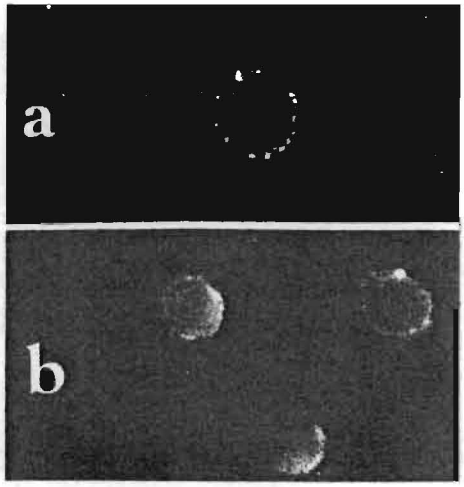

Fig. 1 a. Lymphocyte from a CLL patient showing rim membrane immunofluorescence of microaggregates.
b. Capping of T lymphocytes from the same patient.

rabbit control serum, with rabbit anti-human albumin or in blocking experiments. The percentage of stained control lymphocytes was < 4 per cent. With enriched T lymphocytes from CLL patients, a significantly increased percentage of positive stained cells could be observed (Table 1). The percentage of positive stained eariched B cells was between 5 and 7 per cent. No more than 3-4 per cent of fluorescent stained cells could be observed on enriched T or B control lymphocytes.

E rosettes with SRBCs

After preincubation with anti-AFP antibody an increase in the E-rosette-forming capacity of the patients' lymphocytes could be observed in seven out of 12 patients when compared with the same lymphocytes treated in medium only (Table 1). A prolonged incubation at 4°C with SRBCs seemed to be favourable for additional enhancement of the E-rosette-forming capacity of anti-AFP-treated CLL lymphocytes (Table 2). The E-rosette-forming capacity of control lymphocytes was not affected by anti-AFP treatment at various concentrations or by prolonged incubation with SRBCs (Table 2).

Table 2 Effect of prolonged incubation at 4°C on the E-rosette formation of anti-AFP-treated lymphocytes from controls and CLL patients.

Lymphocytes	No. of cases	E rosettes (%) after 24 hours of incubation		
		Untreated cells	Treated cells	
			Medium	Anti-AFP*
Controls	7	56.4 ± 5.2**	52.2 ± 4.1	56.2 ± 5.1
CLL	7†	11.8 ± 2.6	14.4 ± 4.0	23.5 ± 5.3

†Patients 2,3,7,8,10,11,12 from Table 1.
**Mean % ± SE.
*A dilution of 1:200 anti-AFP was found optimal for the modulating effect on E-rosette-forming lymphocytes from CLL patients. Control cells were not affected by pretreatment with anti-AFP (1:20, 1:100, 1:200). Student's paired "t" test for the evaluation of the anti-AFP effect. (Anti-AFP treated vs. untreated lymphocytes, $p < 0.05$).

T lymphocytes with Fc IgG receptors

As can be seen in Figure 2, the percentage of Fc IgG receptor bearing T lymphocytes was significantly increased in CLL patients compared with controls. After preincubation with anti-AFP antibody, the percentage of Fc IgG receptor bearing T lymphocytes from CLL patients was significantly lower than in those without treatment and was comparable to the percentage of Fc IgG receptor bearing T lymphocytes from untreated controls.

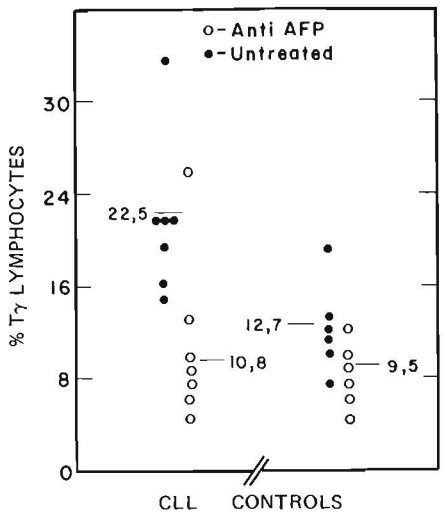

Fig. 2 Tγ lymphocytes in CLL patients and controls. Effect of anti-AFP. For CLL patients, Tγ significantly decreased after antibody treatment. ($p<0.01$). No significant difference for controls. (Student's paired "t" test).

DISCUSSION

The indirect immunofluorescence technique used in this study revealed that AFP antigen can be detected as a surface-binding antigen on some circulating lymphocytes from CLL patients. The increased percentage of stained cells in the T-cell enriched lymphocyte population of CLL patients, the low percentage of such cells in the B-cell-enriched population, the modulating effect of anti-AFP treatment on some T-lymphocyte surface markers of these patients, on spontaneous E-rosette formation and on the T-lymphocyte Fc IgG receptor, seem to suggest that AFP antigen is mainly bound to the cell surface of certain T-lymphocyte subpopulations. Although it remains unclear from this study how AFP antigen-antibody complexes can interfere with the E-rosette-binding lymphocyte receptor, it may be assumed that movement on the cell surface of AFP antigen-antibody complexes and topographic redistribution of membrane components may lead to the unmasking of the specific E-rosette receptor and to an increased binding capacity of these lymphocytes to SRBCs.[17] A prolonged incubation with SRBCs at 4°C was observed to enhance further the number of probably previously cryptic or unavailable E-rosette-binding receptor sites of anti-AFP-treated lymphocytes from CLL pa-

tients. No such enhancement could be observed with control lymphocytes.

Our observation on an increased percentage of Tγ cells in CLL patients correlates well with the observation of Gupta and Good[14] concerning increased number of Tγ cells in patients with certain types of immunodeficient disorders. Murgita *et al*[2] observed that AFP can induce suppressor T cells *in vitro*. The modulating effect of anti-AFP on the Fc IgG receptor of T lymphocytes from CLL patients observed in this study and the reduced expression of Fc IgG receptors after anti-AFP treatment suggest various explanations. These phonomena may be due to an inhibitory effect,[18] but may also be the result of a close location of the Fc IgG-complex receptor and AFP antigen binding sites on the same lymphocyte membrane surface.

AFP binding sites were suggested to be closely spaced to Con A receptor sites on murine T lymphocytes.[1] Further studies on the modulating effect of anti-AFP antibody on additional surface markers of lymphocytes from CLL patients and examinations of some immune functions of AFP-bearing lymphocytes seem to be necessary.

REFERENCES

1. Dattwyler RJ, Murgita RA, Tomasi TB Jr. Binding of alpha-fetoprotein to murine T cells. Nature 1975; 256:656-7.
2. Murgita RA, Goidl E, Kontiainen S, Wigzell H. Alpha fetoprotein induces suppressor T cells *in vitro*. Nature 1977; 267:257-9.
3. Gupta S, Good RA. α fetoprotein and human lymphocyte subpopulations. J Immunol 1977; 118:405-8.
4. Murgita RA, Anderson LC, Sherman MS, Bentwich H, Wigzell H. Effects of human alpha-fetoprotein in human B and T lymphocyte proliferation *in vitro*. Clin Exp Immunol 1978; 33:347-56.
5. Chism SE, Order SE, Hollman S. Tumor-fetal antigens in Hodgkin's disease: an immunoelectrophoretic analysis. Am J Roentgenol Radium Ther Nucl Med 1973; 117:5-11.
6. Moroz C, Giler S, Kupfer B, Urca I. Lymphocytes bearing surface ferritin in patients with Hodgkin's disease and breast cancer. N Engl J Med 1977; 296:1172-3.
7. Mukherji B. Hirschaut Y. Evidence for fetal antigen in human sarcoma. Science 1973; 181:440-2.
8. Pathouli Ch, Karvountzis G, Stergiou A, Hadziyannis S, Komninos Z. AFP-positive lymphocytes in lymphoproliferative disorders, hypogammaglobulinemias and liver diseases. Abstracts of the Meeting on Immunological Aspects of Experimental and Clinical Cancer, October 28 – November 1, 1979, Tel-Aviv, p. 122.
9. Bentwich S, Cohen I, Sulitzeanu D, Iszak G, Weiss DW. Antigenicity of lymphocytes from chronic lymphocytic leukemia (CLL) patients and from normal newborns. Proceedings of the American Association of Cancer Research. 1970; 2:7.
10. Anderson LC, Wasastjerna C, Calemberg CG. Different surface glycoprotein patterns on human T-, B-, and leukemic-lymphocytes. Int J Cancer 1976; 17:40-6.
11. Rundles WR. Chronic lymphocytic leukemia. In: Williams WJ, Beutler E, Erslev AJ, Rundles RW, eds, Hematology. 2nd ed. New York: McGraw Hill, 1977: 1004.
12. Komlos L, Halbrecht I, Shachor J, Djaldetti M. The effect of sera from chronic lymphocytic leukemia patients on normal mitogen-stimulated lymphocytes. A possible correlation with the patients' T and B lymphocytes. Exp Hematol 1978; 6: 595-600.
13. Ray KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternak BS. Clinical staging of chronic lymphocytic leukemia. Blood 1976; 46:219-34.
14. Gupta S, Good RA. Subpopulations of human T lymphocytes. I. Studies in immunodeficient patients. Clin Exp Immunol 1977; 30:222-8.
15. Boyum A. Separation of leukoyctes from blood and bone marrow. Scand J Clin Lab Invest 1968; Suppl 97:31-5.
16. Bentwich A, Douglas SD, Siegal FP, Kunkel HG. Human-lymphocyte-sheep erythrocyte formation: some characteristics of the interaction. Clin Immunol Immunopathol 1973; 1:511-22.
17. Speckart SF, Boldt DH, Ryerson KL. Cell surface changes in transformed human lymphocytes. I. Con A and E-PHA induced unique changes in surface topography. Exp Cell Res 1978; 111:385-95.
18. Rhodes J. Modulation of macrophage Fc receptor expression in vitro by insulin and cyclic nucleotides. Nature 1975; 257:597-9.

Demonstration of Anti-asialo GM1 Antibody and Its Neurocytotoxicity in the Sera of Systemic Lupus Erythematosus Patients*

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SUMMARY **The presence of anti-asialo-GM1 antibody and its neurocytotoxicity in the sera of patients with SLE were studied. The antibody was detected in 55 per cent of patients with CNS complications. but not in healthy adult controls nor in patients without CNS complications. The exacerbation of CNS symptoms of CNS-SLE patients was accompanied by elevation of the anti-GA1 antibody level in the sera. In the presence of complement, the anti-GA1 antibody in the patients' sera showed cytotoxicity against nervous tissue cells bearing GA1 on their surface. The roles of these antibodies in the pathogenesis of CNS-SLE are suggested.**

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As much as 30 to 50 per cent of patients with systemic lupus erythematosus (SLE) have central nervous system (CNS) complications.[1] Following renal insufficiency, CNS complications are the second most frequent cause of death of SLE patients and thus are deeply related to the pathogenesis of SLE. However, the pathogenesis of CNS complications has not been fully clarified and so diagnosis and treatment are still in dispute.

Since the early 1970s, there has been considerable interest in antineuronal antibodies in the sera and cerebrospinal fluid (CSF) of SLE patients in relation to the pathogenesis of CNS complications.[2-18] For example, antineuronal antibodies,[2,3,7,9,10] anti-glial antibodies,[7] anti-lymphocytes antibodies cross-reactive with brain tissue,[5,6] antibodies against the antigenic determinants shared between erythrocytes and the brain[9] and anti-glycolipid antibodies[11,19] have been reported to exist in the sera of SLE patients. Anti-brain tissue antibodies and IgG neurocytotoxic antibodies also have been demonstrated in CSF of SLE patients.[12,13] Recently, antineuronal antibodies of the IgG and IgA classes have been shown to exist in the necropsy brain tissue of an SLE patient with CNS complication (CNS-SLE).[16] Implantation of antibodies directed against various brain constituents into the ventricles or cerebral cortex of experimental animals caused convulsion, meningitis, impaired memory or motor dysfunction.[20-22] These observations suggest that antineuronal antibodies may play an important role in the CNS complications of SLE. Antineuronal antibodies in the sera of SLE patients have been recognised primarily by the immunofluorescence technique; hence, the physicochemical characteristics of the corresponding antigens have not been clarified. However, Hirano *et al*[11] detected antibody against the neutral glycolipid "asialo GM1 (GA1)" (ganglio-N-tetraosyl ceramide) in the sera of CNS-SLE patients. We also observed that exacerbation of CNS symptoms of a 10-year-old female CNS-SLE patient correlated with the elevation of the titre of the serum anti-GA1 antibody.[19]

Since GA1 exists in trace amounts in the human brain, anti-GA1 antibody could be one of the antineuronal antibodies in the sera of SLE patients. In the present

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study, we examined the neurocytotoxicity of anti-GAl antibody using established tissue culture cells derived from human brain tumours as targets.

MATERIALS AND METHODS

Patients and sera

Sera were obtained from 30 SLE patients (including 20 CNS-SLE patients) and 20 healthy adult controls and stored without preservative at -70°C until use. All the SLE patients met the criteria of SLE of the American Rheumatism Association. The criteria of neurologic disorders in SLE included definite neurologic (both central and peripheral) or psychiatric abnormalities not attributed to non-SLE aetiologies.

Cultured cell lines derived from human brain tumours

IMR-32 (human neuroblastoma)[23] 118 MGC (human glioblastoma)[24] and KG-1 (human oligodendroglioma)[25] were used as the targets of cytotoxicity assays. Cells were maintained in continuous culture in Minimal Essential Medium (Eagle's MEM, Nissui Seiyaku Co., Ltd. Tokyo, Japan) supplemented with L-glutamine and 5% foetal bovine serum. Viable cell suspensions were prepared by scraping adhesive cells with a rubber-policeman and suspending them in phosphate buffered saline (PBS: 8.0 g of NaCl, 0.2 g of KCl, 2.9 g of Na_2HPO_4 $12H_2O$ and 0.2 g of KH_2PO_4 in 1,000 ml of distilled water). They were used for tests when the viability was above 90 per cent.

Preparation of GAl

GMl was isolated from bovine brain tissue by a combination of anion-exchange chromatography and high performance adsorption chromatography.[26] GAl was prepared from GMl by treatment with 1N-formic acid at 100°C for one hour[27] and then purified by chromatography on DEAE-Sephadex A-25 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and Silica Gel 40 (Merck & Co., Inc., Rahway, N.J., U.S.A.).[28]

Preparation of antisera against GAl

Liposomes containing 1 mg of GAl, 2 mg of bovine serum albumin (BSA), 4 mg of cholesterol and 10 mg of lectithin were prepared and suspended in 0.5 ml of PBS. The same volume of Freund's complete adjuvant (Difco Lab., Detroit, Michigan, U.S.A.) was added to the suspension and the mixture was emulsified. Te emulsion was administered to a rabbit by intracutaneous injection at several sites on the foot-pads. Two to three weeks after the injection, blood with high titre anti-GAl antibody was obtained, and the plasma was heat inactivated (56°C for 30 minutes) and stored at -70°C with 0.05% sodium azide.

Detection of anti-GAl antibody

An enzyme linked immunosorbent assay (ELISA) was used.[29] A 50-μl volume of GAl solution (10μl per ml of 0.05% deoxycholate-PBS) was added to each well of Linbro E.I.A. 96-well flat-bottom microtitration plates (Flow Lab Inc, Maclean, Virginia, U.S.A.) and the plates were stored at 4°C overnight. After coating the plate with GAl in this way, sera serially diluted in 0.05% Twen 20 (v/v)-2% BSA (w/v)-PBS (dilution buffer) was added to each well. After incubation at room temperature for two hours, each well was washed three times with 0.05% Tween 20 (v/v)-PBS (washing buffer). A 50-μl volume of peroxidase-labelled anti-human IgG (Cappel Lab, Inc., U.S.A.) diluted 1:200 in dilution buffer was added to each well and the plate was incubated at room temperature for one hour. Excess conjugate was washed out and 160 μl of enzyme substrate solution was added; the latter was composed of 10 volumes of an aqueous solution containing 2,2'-azino-di (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (15 mg/ml), 1,000 volumes of citric acid monohydrate solution (10.5 mg/ml) adjusted to pH 3.9-4.1 with 5N-NaOH and 33 volumes of 35% hydrogen peroxide. After incubation at room temperature for 15 minutes, the reaction was stopped with the addition of 160 μl of stop solution, composed of 100 volumes of solution A (0.347 ml of 50% HF and 0.2 ml of 3N-NaOH in 100 ml of distilled water) and 1 volume of another solution B (2.92 g of EDTA and 1.6 g of NaOH in 100 ml of distilled water). The absorbance at 414 nm of the content of each well was measured against distilled water as a blank. As a control for each serum, we measured the absorbance of uncoated wells to which were added with patient's serum diluted 1:10. The antibody titre was determined by the end-point of the serum dilution giving an enzyme activity higher than that of each control.

Preparation of the IgG fraction from SLE patients' sera

Each patient's serum was applied to a Protein A-Sepharose CL-4B affinity column (Pharmacia Fine Chemicals AB, Uppsala, Sweden). After elution of IgG bound to the column by 10 bed volumes of 1M – acetic acid, the eluate was dialysed, concentrated by AMICON-15 filtration to the same volume as the original serum and used as the IgG fraction.

Absorption of anti-GAl antibody

Anti-GAl antibody was absorbed from the sera or their IgG fraction by incubating the samples overnight at 4°C in microtitration plates coated with GAl.

Cytotoxicity of the IgG fraction of patients' sera and rabbit anti-GAl serum against cells of neural origin

Cultured cells which originated from a human neuroblastoma (IMR-32), a human glioblastoma (118 MGC) and a human oligoden-

droglioma (KG-1) were dislodged with a rubber policeman, dissociated with a Pasteur pipette and washed once with PBS. The cells were suspended in PBS at a concentration of $4x10^6$/ml. A 25 μl aliquot of each cell suspension was mixed with 50 μl of each patient's serum, IgG fraction or with rabbit anti-GAl serum diluted 1:10 in PBS. After incubation at room temperature for 30 minutes, 50 μl of fresh rabbit serum diluted 1:10 in PBS was added as a source of complement and then incubated at 37°C for 30 minutes. The reaction was stopped in ice bath. The 100 μl of 0.5% trypan blue-0.85% saline solution was added to each tube and the percentage of dead cells was calculated under a microscope. Dead cells in controls amounted to less than 10 per cent in all experiments. The cytotoxicities of the sera and their IgG fractions from which anti-GA1 antibody was eliminated were also examined.

Detection of GAl on the surface of neural cells by the indirect immunofluorescence method

By the above technique, $5x10^5$ cultured cells were suspended in PBS and pelleted by centrifugation (150xg for five mintues). A 50-μl volume of rabbit anti-GAl serum diluted 1:10 in PBS was added to each pellet and incubated at room temperature for 45 minutes. Cells were washed twice in PBS, and 50 μl of FITC-labelled goat anti-rabbit IgG (Cappel Lab, Inc., U.S.A.) diluted 1:5 in PBS was added and incubated at 4°C for 30 minutes. After again washing twice with PBS, another pellet was made by centrifugation. The pellet was dispersed in 50μl of 50% glycerine-PBS with a Pasteur pipette, and was put on a glass slide and observed under a fluorescence-microscope. As controls, pre-immune rabbit serum, rabbit anti-GAl serum after elimination of anti-GAl antibody and rabbit anti-BSA antibody were also used as reagents.

RESULTS

Anti-GAl antibody titres of the sera from patients with SLE

The anti-GAl antibody titres of sera from 30 patients, including 20 cases with CNS-SLE, were examined. Anti-GAl antibody titres of 1:40 or above were detected in 55 per cent of the patients with CNS-SLE. In all of the sera from the SLE patients without CNS complications and from 20 healthy adult, anti-GAl antibody titres were 1:20 or less (Table 1).

The anti-GMl antibody titres of sera from 24 SLE patients, including 14 cases with CNS-SLE, were also examined. In all of the sera from the SLE patients with or without CNS complications, anti-GMl antibody titres were 1:20 or less (Table 2).

Anti-GAl antibody titres and the CNS complications of SLE

In the observation of two cases of CNS-SLE, the exacerbation of CNS symptoms was accompanied by the elevation of the anti-GAl antibody titre (Fig. 1).

The correlation between anti-GAl antibody titres and their cytotoxicities against the cultured neural cells of the sera from SLE patients or rabbit anti-GAl serum

The sera of CNS-SLE patients having high titres of anti-GAl antibody and rabbit anti-GAl serum were strongly cytotoxic to IMR-32 cells. On the other hand, the sera from SLE patients without CNS complications and from healthy adults showed no cytotoxicity (Fig. 2). However, neither anti-GAl antibody-rich sera of CNS-SLE patients nor rabbit anti-GAl serum showed cytotoxicity to 118 MGC cells or KG-1 cells (data not shown).

The cytotoxicity against IMR-32 cells of the sera of SLE patients and rabbit anti-GAl serum after elimination of anti-GAl antibody

After the absorption of anti-GAl

Table 1 Anti-GA_1 antibody titre in the sera of 30 patients with SLE

	Cases	Anti-GA_1 antibody titre				Total positive %
		< x 20	x 20	x 40	x 80 <	
SLE with CNS involvement	20	3	6	3 (15.0%)	8 (40.0%)	55.0
SLE without CNS involvement	10	8	2	–	–	0.0
Healthy adult	20	20	–	–	–	0.0

Table 2 Anti-GM_1 antibody titre in the sera of 24 patients with SLE

	Cases	anti-GM_1 antibody titre				Total positive %
		< x 20	x 20	x 40	x 80 <	
SLE with CNS involvement	14	13	1	–	–	0.0
SLE without CNS involvement	10	8	2	–	–	0.0
Healthy adult	20	20	–	–	–	0.0

antibody, rabbit anti-GAl serum lost completely its cytotoxicity against IMR-32 cells. The sera of CNS-SLE patients lost most of the activity in that regard, although slight cytotoxicity remained (Fig. 3).

Cytotoxicity against IMR-32 cells of the CNS-SLE serum IgG fraction

The IgG fractions were separated from the anti-GAl antibody-rich sera of CNS-SLE patients. The serum IgG fraction had as much cytotoxicity against IMR-32 cells as the original sera (Fig. 4).

The cytotoxicity against IMR-32 cells of the CNS-SLE serum IgG fraction, from which anti-GAl antibody had been absorbed, did not show any cytotoxicity against IMR-32 cells (Fig. 5).

Staining of neural cells for GAl antigen by immunofluorescence

Almost all the IMR-32 cells were fluorescence-stained on their cell surface membrane after reaction with rabbit anti-GAl serum (data not shown). However, no fluorescence was detected on the cell membrane of 118 MGC cells nor KG-1 cells. When the rabbit preimmune serum or rabbit anti-GAl serum with the anti-GA1 antibody removed by absorption, were used as reagents, no fluorescence was detected on the surface of these three cells lines.

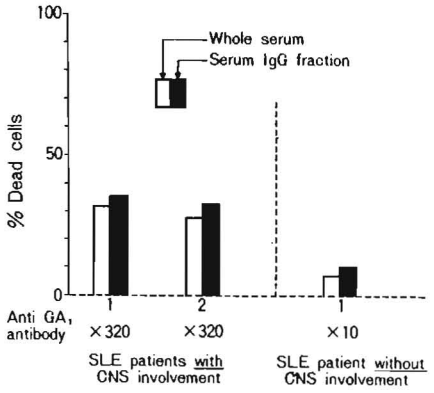

Fig. 4 Comparison of cytotoxicity against IMR-32 cells between whole serum and serum IgG fraction of SLE patients with and without CNS involvement

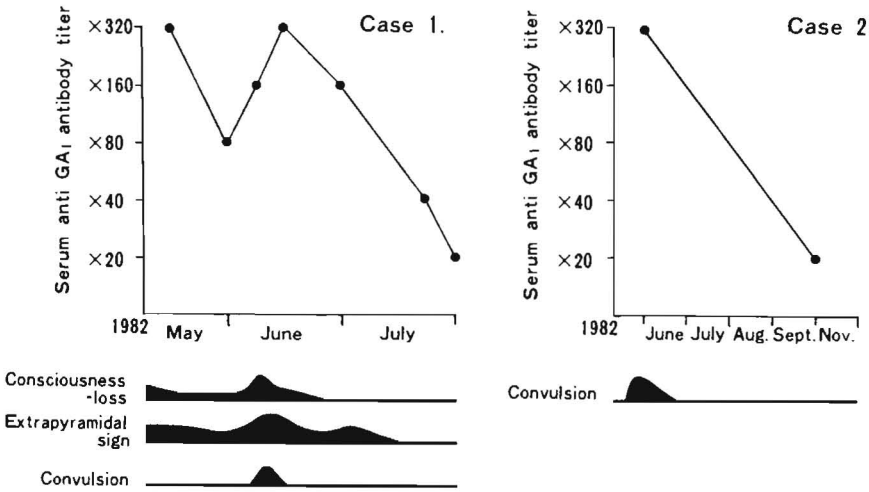

Fig. 1 Clinical course and anti-GA_1 antibody titre in 2 CNS-SLE patients.

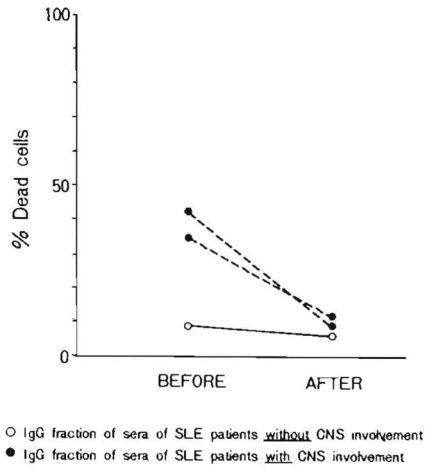

Fig. 5 Alteration of cytotoxicity of IgG fraction against IMR-32 cells after elimination of anti-GA_1 antibody

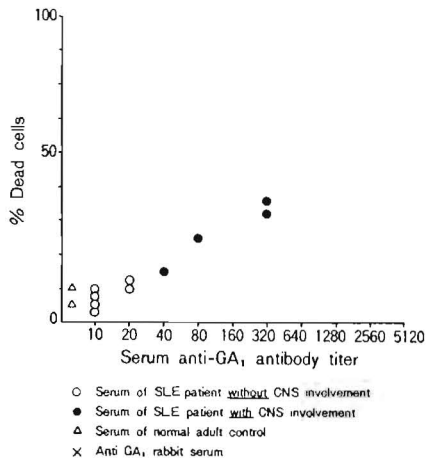

Fig. 2 Relationship between cytotoxicity of sera against IMR-32 cells and serum anti-GA_1 antibody titre

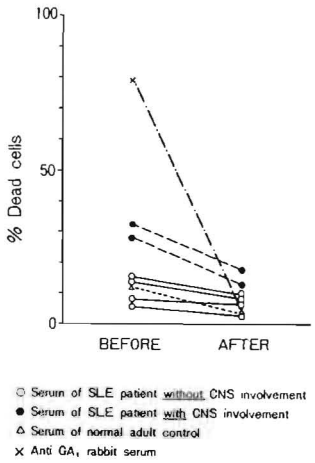

Fig. 3 Alteration of cytotoxicity of sera against IMR-32 cells after elimination of anti-GA_1 antibody

DISCUSSION

It was shown in this study that the sera of CNS-SLE patients had cytotoxic activity against IMR-32 cells (a cell line derived from a human neuroblastoma) and that the activity was reduced after the elimination of anti-GAl antibody from the sera. These results suggest that serum anti-GAl antibody of CNS-SLE patients has a role to play in the cytotoxicity. In contrast to rabbit anti-GAl serum, which lost its cytotoxicity completely after the absorption of anti-GAl antibody, the sera of SLE patients retained activity to some extent after the absorption of anti-GAl antibody. This indicates that some unknown neurocytotoxic factors other than anti-GAl antibody could be present in the sera of SLE patients. Because anti-GAl antibody had been reported to be mainly of the IgG class,[11] we exmained the cytotoxicity of the IgG fractions of the sera. A large proportion of the IgG neurocytotoxic antibodies in CNS-SLE sera was determined to be anti-GAl antibody in that the original sera and their IgG fractions showed almost the same cytotoxicity; IgG fractions with the anti-GA1 antibody removed were no longer cytotoxic against IMR-32 cells. Bluestein *et al* reported that 75 per cent of SLE sera had cytotoxic activity to SK-N-SH cells, a line also of human neuroblastomal origin, and that this cytotoxicity derived from IgG and/or IgM antibodies.[7] Our results were consistent with their report. It should be emphasised that, in the present study, one of the most important antigens reacting with antineuronal antibodies in the SLE sera was demonstrated to be an unique chemical substance, GAl. The SLE sera were not cytotoxic against 118 MGC cells and KG-1 cells probably because of the absence of GAl on their cell membrane. Using as targets three cell lines of neural origin (SK-N-SH, LA-N-1 and IMR-32) and two of glial origin (A-172 and U-118 MG), Bluestein *et al* demonstrated that 40 per cent of the SLE sera were cytotoxic to all five cell lines and 25 per cent reacted against at least one neural and one glial cell line, while 15 per cent had antineural but not anti-glial reactivity.[8] The differences between their conclusions and ours may arise from the different cell lines, sera and/or assays used.

IMR-32 cells were determined to have GAl on their surface, because they were fluorescein-stained by rabbit anti-GAl serum but not by pre-immune rabbit serum or rabbit anti-GAl serum with the anti-GAl antibody removed by absorption. The unequivocal identification of the antigenic substance of these cells as GAl requires further biochemical analysis. Our preliminary biochemical analysis using thin-layer chromatography suggested that the substance was truly GA1 (data not shown). On the other hand, 118 MGC cells and KG-1 cells showed no immunofluorescence and therefore, the absence of GAl on these cells was suggested.

Anti-GAl antibody would be cytotoxic only to GAl-positive cells among the various nerve tissue cells. In order for the circulating anti-GAl antibody to be pathogenetic to CNS tissues, destruction of the blood-brain barrier would be required to allow the antibody to enter the CNS so as to bind directly with brain tissues. In this regard, we demonstrated anti-GAl antibody in the CSF of two cases of CNS-SLE patients (in preparation). Bluestein *et al* similarly found that CSF from CNS-SLE patients had elevated IgG antineuronal activity.[13] As previously mentioned, it was reported that antineuronal antibody (IgG or IgA) was bound directly to brain tissue in one autopsied CNS-SLE case.[16]

In terms of correlation between antineuronal antibody and CNS symptoms of SLE, some authors have reported affirmateively[3,9,10] while others negatively.[17] This is probably due to the fact that antineuronal antibodies are heterogeneous. In our investigation, the exacerbation of CNS symptoms was accompanied by the elevation of the titre of serum anti-GAl antibody. These results indicated that anti-GA1 antibody titre in the CNS-SLE sera could be a good indicator of its CNS involvement.[19] Hirano *et al* reported that anti-GAl antibody decreased concurrently with periods of convulsive disorders and the titres recovered some time later.[11] This discrepancy between their results and ours probably arises from the difference in the assays used.

Recently, elevation of anti-GAl antibody was demonstrated also in the sera of patients with Neuro-Behcet's disease and AIDS (acquired immune deficiency syndrome.)[30] Only low titre of anti-GAl antibody in the sera of neurological diseases other than CNS-SLE and autoimmune diseases was detected in our studies (in preparation). Anti-GM1 antibody was not found in the sera of our CNS-SLE patients; antibodies to asialo GM2 and to galactocerebroside were reported not to exist in the sera of CNS-SLE patients.[11] Therefore, the marked elevation of anti-GAl antibody titre in the sera was rather characteristic of CNS-SLE.

GAl is known to be an antigen shared by immunocytes and nervous tissues. Sera of rabbits immunised with mouse brain homogenate have marked anti-Natural Killer (NK) activity and contain high titres of anti-GAl antibody. In the presence of complement, anti-GAl antibody inhibits mouse NK activity and this anti-NK activity is eliminated after absorption with mouse brain homogenate or GAl.[31-33] These findings indicate that, in mice, GAl is common to NK cells and nervous tissues. GAl was reported to exist on the surface of suppressor T cells as well as NK cells in mice,[34] but it has not been demonstrated to be on the surface of guinea pig or human NK cells. In rats, GAl was demonstrated on

the surface of thymocytes, peripheral T cells, granulocytes and macrophages.[35] The distribution of GA1 thus seems quite different from species to species. A small amount of GA1 is derived from human brain tissue, but the distribution of GA1 in nervous tissues is not yet understood. The existence and the distribution of GA1 on the surface of human lymphocytes have been in dispute. Normally, human lymphocytes are thought not to have GA1 on the surface, but lymphocytes from thymoma patients with myasthenia gravis or acute lymphoblastic leukaemia cells are reported to have GA1 on their surface.[36] Shinomiya *et al* reported that Con A-induced suppressor activity was inhibited by anti-GA1 antibody in the presence of complement,[37] presumably indicating that suppressor T cells induced by Con A possessed some substance which reacted with anti-GA1 antibody.

Although the distribution of GA1 and the effects of anti-GA1 antibody on the host are thought to be quite different among humans conpared with other species, GA1 seems to be an antigen common to both immunocytes and nervous tissues in various animals. The definition of the effects of anti-GA1 antibody may lead to great progress in clarifying the pathogenesis of neuroimmunological disorders.

REFERENCES

1. Dubois EL, Tuffanelli DL. Clinical manifestation of systemic lupus erythematosus-computer analysis of 520 cases. JAMA 1964; 1920:112-9.
2. Diederichsen H, Pyndt IC. Antibodies against neurons in a patient with systemic lupus erythematosus, cerebral palsy and epilepsy. Brain 1970; 93:407-12.
3. Quismorio FP, Friou GJ. Antibodies reactive with neurons in systemic lupus erythematosus patients with neuropsychiatric manifestations. Int Arch Allergy 1972; 43:740-8.
4. Bluestein HG, Zvaifler NJ. Brain-reactive lymphocytotoxic antibodies in the serum of patients with systemic lupus erythematosus. J Clin Invest 1976; 57:507-16.
5. Bluestein HG. Antineuronal activity in systemic lupus erythematosus (SLE) serum. Arth Rheum 1977; 20:109.
6. Bresnihan B, Oliver M, Grigor R, Hughes GRV. Brain reactivity of lymphocytotoxic antibodies in systemic lupus erythematosus with and without cerebral involvement. Clin Exp Immunol 1977; 30:333-7.
7. Bluestein HG. Neurocytotoxic antibodies in serum of patients with systemic lupus erythematosus. Proc Natl Acad Sci USA 1978; 75:3965-9.
8. Bluestein HG. Heterogeneous neurocytotoxic antibodies in systemic lupus erythematosus. Clin Exp Immunol 1979; 35: 210-7.
9. Bresnihan B, Oliver M, Williams B, Hughes GR. An antineuronal antibody cross-reacting with erythrocytes and lymphocytes in systemic lupus erythematosus. Arth Rheum 1979; 22:313-20.
10. Wilson HA, Winfield JB, Lahita RG, Koffler D. Association of IgG antibrain antibodies with CNS dysfunction in systemic lupus erythematosus. Arth Rheum 1979; 22:458-62.
11. Hirano T, Hashimoto H, Shiokawa Y, et al. Antiglycolipid autoantibody detected in the sera from systemic lupus erythematosus patients. J Clin Invest 1980; 66: 1437-40.
12. Williams GW, Bluestein HG, Steinberg AD. Brain-reactive lymphocytotoxic antibody in the cerebrospinal fluid of patients with systemic lupus erythematosus: correlation with central nervous system involvement. Clin Imm Immunopath 1981; 18:126-32.
13. Bluestein HG, Williams GW, Steinberg AD. Cerebrospinal fluid antibodies to neuronal cells: association with neuropsychiatric manifestations of systemic lupus erythematosus. Am J Med 1981; 70:240-6.
14. Bluestein HG, Zvaifler NJ. The pathogenesis of central nervous system manifestations of systemic lupus erythematosus. Arth Rheum 1982; 25:862-6.
15. Bluestein HG, Woods VL Jr. Antineuronal antibodies in systemic lupus erythematosus. Arth Rheum 1982; 25:773-8.
16. Inoue T, Okamura M, Amatsu K, Olie A, Kato N, Kanayama Y. Antineuronal antibodies in brain tissue of patients with systemic lupus erythematosus. Lancet 1982; 2:852.
17. Pussel BA, Blyth F, Charlesworth JA. Failure to detect brain reactivity of lymphocytotoxins in cerebral lupus. Clin Exp Immunol 1982; 47:133-7.
18. Tamesvari P, Denburg S, Carbotte R, Bensen W, Singel D. Serum lymphocytotoxic antibodies in neuropsychiatric lupus: a serial study. Clin Imm Immunopath 1983; 28:243-51.
19. Hoshino C, Shinomiya N, Yata J, et al. A case of CNS-SLE dramatically improved by PULSE therapy-anti-asialo GM1 antibody in the course. Acta Pediatr Japon 1984; 26:103.
20. Simon J, Simon O. The effect of passive transfer of antibrain antibodies to a normal recipient. Exp Neurol 1975; 47:523-34.
21. Kobiler D, Fuchs S, Samuel D. The effect of antisynaptosomal plasma membrane antibodies on memory. Brain Res 1976; 115:129-38.
22. Rapport MM, Karpiak SE, Mahadik SP. Biological activities of antibodies injected into the brain. Fed Proc 1978; 38:2391-6.
23. Tumilowicz JJ, Nichols WW, Cholon JJ, Greene AE. Definition of a continuous human cell line derived from neuroblastoma. Cancer Res 1970; 30:2110-8.
24. Ponten J, Macintyre EH. Long term culture of normal and neoplastic human glia. Acta Path Microbiol Scandinav 1968; 74: 465-86.
25. Miyake E. Establishment of a human oligodendroglial cell line. Acta Neuropathol 1979; 46:51-5.
26. Momoi T, Ando S, Nagai Y. High resolution preparative column chromatographic system for ganglioside using DEAE-sephadex and a new porus silica, Iatrobeads. Biochim Biophys Acta 1976; 441:488-97.
27. Svennerholm L, Mansson JE, Li YT. Isolation and structural determination of a novel ganglioside, a disialosylpentahexosyl ceramide from human brain. J Biol Chem 1973; 248:740-2.
28. Taki T, Hirabayashi Y, Takagi K, Kamada R, Kojima K, Matsumoto M. Purification of anti-glycosphingolipid antibody and topological localization of glycosphingolipid on the cell surface of rat ascites hepatomas. J Biochem 1981; 89:503-10.
29. Irie H, Sanai Y, Nagai Y. Enzyme-linked immunosorbent assay (ELISA) for anti-myelin basic protein antibody and its application of studies of experimental allergic encephalitis. Japan J Exp Med 1981; 51:201-8.
30. Witkin SS, Sonnabent J, Richards JM, Purtilo DT. Induction of antibody to asialo GM1 by spermatozoa and its occurrence in the sera of homosexual men with the acquired immunodeficiency syndrome (AIDS). Clin Exp Immunol 1983; 54:346-50.
31. Habu S, Hayakawa K, Okumura K, Tada T. Surface markers on natural killer cells of the mouse. Eur J Immunol 1979; 9: 938-42.
32. Kasai M, Iwamori M, Nagai Y, Okumura K, Tada T. A glycolipid on the surface of mouse natural killer cells. Eur J Immunol 1980; 10:175-80.
33. Young WW, Hakomori S, Durdik JM, Henney CS. Identification of ganglio-N-tetraosylceramide as a new cell surface marker for murine natural killer (NK) cells. J Immunol 1980; 124:199-201.

34. Nakano T, Imai Y, Naiki M, Osawa T. Characterisation of mouse helper and supressor T cell subsets separated by lectins. J Immunol 1980; 125:1928-32.
35. Momoi T, Wiegandt H, Arndt R, Thiele HG. Gangliotetraosyl-ceramide, the rat T lymphocyte-macrophage-associated antigen: chemical detection and cellular distribution. J Immunol 1980; 125:2496-500.
36. Nakahara K, Ohashi T, Oda T, et al. Asialo GM1 as a cell-surface marker detected in acute lymphoblastic leukemia. New Engl J Med 1980; 302:674-7.
37. Shinomiya N. Antibodies to surface membrane antigen expressed on lymphocytes and their function. Clin Immunol 1982; 14:881-8.

Beclomethasone dipropionate and Flunisolide: an Open-crossover Comparative Trial in Perennial Allergic Rhinitis*

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SUMMARY **Flunisolide nasal solution which is a newly synthesised topical corticosteroid was compared with a well-documented beclomethasone dipropionate aerosol by an open-crossover trial in a group of 45 perennial rhinitis patients.**

Both flunisolide and BDA have been shown to effect significant control of itching, sneezing, stuffy nose and running nose. In both groups, there were no significant changes of the absolute eosinophil count, the number of eosinophils in the nasal smear, and the nasal swabs for bacteria and fungi. But the symptom scores rated after the nasal provocative test decreased significantly after each treatment. The total serum IgE level increased in both groups, but it was statistically significant only in the flunisolide group. The side-effects were reported more frequently in the group of flunisolide users but most of them were mild. The physicians' and patients' opinions about the effectiveness of each treatment were similar although the overall changes in mean symptom scores of all symptoms and the patients' preference favoured the use of BDA. We concluded that flunisolide is probably a valuable alternative to BDA when perennial rhinitis requires treatment with a topical corticosteroid.

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Beclomethasone dipropionate aerosol (BDA) has since 1973 been used intranasally in treating patients with hay fever.[1] Its efficacy has also been proved in our perennial allergic rhinitis patients.[2] At present, another new potent topical corticosteroid, "flunisolide", which is closely related to fluocinolone acetonide, has been introduced for the treatment of patients with allergic rhinitis. Many clinical trials on the use of flunisolide nasal spray have demonstrated its superiority to a placebo for the treatment of perennial rhinitis[3-8] and seasonal allergic rhinitis.[8-10] A similar result was also observed in children with a lack of any serious side-effects.[11,12] Therefore, it may be concluded that BDA and flunisolide are safe and effective steroid analogues for use intranasally in treating cases of nasal allergy. A parallel comparison between the effectiveness of BDA and flunisolide has been performed; it showed that both of them are equally effective.[13] However, our clinical study to compare the effectiveness of BDA and flunisolide in perennial rhinitis patients was carried out in an open-crossover design to evaluate the superiority of these two preparations in order to use them properly.

MATERIALS AND METHODS

Forty-eight patients were involved in this study. They were all attending the ENT Allergy Clinic of Siriraj Hospital for treatment of their perennial rhinitis symptoms. Only 45 patients (30 females and 15 males) completed the study. Their ages ranged from 16 to 57 years; the average age was 28.5 years. The duration of symptoms ranged from one to 22 years, the average duration being 7.3 years. Four patients also had bronchial asthma which was not severe enough for them to use bronchodilators regularly. None had received any form of steroid therapy during the preceding six months. It should be stressed that no BDA had been prescribed previously for this

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group of patients. No patient had any signs nor symptoms of infection in the nose, throat and sinuses at the beginning of and throughout the trial period. A family history of allergic diseases was positive in 34.1 per cent of the patients.

The duration of this trial was eight weeks. Patients were given one kind of treatment for four weeks then another kind for another four weeks. In order to minimise any bias among the physicians who were familiar with BDA from our previous trial, the treatment given to each patient was accomplished on weekly basis by one of our technicians. The physicians who evaluated the results did not know the kind of treatment the patients were being given.

The wash-out period was not designed in this trial because it was stated that the effect of BDA lasted only 1-2 days after it was discontinued.[14] In our own observation, the carry-over effect of flunisolide lasted fewer than 72 hours.

Prior to the commencement of treatment, every patient was tested intracutaneously with a standard panel of 12 common allergenic extracts such as house-dust, house-dust mite, pollens, moulds and household insects. Roentgenography of the chest and paranasal sinuses was also performed; if any abnormality was detected, the patient was excluded from the study. On admission and at the end of each treatment period, every patient had received or undergone the following:

a. a complete routine ENT examination,

b. nasal swabs for bacteriological as well as fungal studies,

c. nasal provocation test with house-dust mite extract.

d. eosinophil estimations of nasal secretions.

e. complete blood count.

f. total serum IgE determination (by Phadebas IgE PRIST test kits).

With regard to BDA, each patient was instructed to inhale one puff in each nostril four times a day for four weeks. This represented a daily dose of 400 micrograms per day.

As for flunisolide, the dosage used was two sprays in each nostril twice daily giving a total daily dose of 200 micrograms of flunisolide per day. This was also done for four weeks.

All patients were also given a kind of antihistamine tablet (chlorpheniramine maleate 4 mg or a combination of tripolidine HCl 2.5 mg and pseudoephedrine HCl 60 mg) to be used supplementarily as necessary.

Patients were assessed on admission to the trial and at the end of each test medication on the following symptoms using a graded scoring system: itching, sneezing, stuffiness and running nose, each rated on a 4-point scale (0= none, 1= slight, 2= moderate, and 3= severe).

Tests for significance of the findings were performed using the Chi-square test, the paired-t test and the student's t test where appropriate.

Earlier studies in experimental animals showed that inhaled flunisolide in doses of less than 4.0 mg per day did not suppress the plasma cortisol levels.[15] Also, investigations on the use of flunisolide in man showed no sign of adrenal suppression at the therapeutic dose.[3-13] There is also sufficient evidence to indicate that long-term use of BDA produces no systemic effect.[1,16-22] Therefore, no test for adrenal function was included in this study.

RESULTS

The 33 patients who received BDA first were allocated to group I and the 12 patients who received flunisolide first were allocated to group II. In the intracutaneous test, every patient showed at least a 2+ reaction to more than two common allergens. By the end of the trial, all patients had used both BDA and flunisolide each for one month and the overall changes of the symptom scores for each symptom after each therapy are shown in Table 1.

Both drug groups showed significant reductions of symptom scores from the control group with regard to itching, sneezing, stuffy nose and running nose. However, when the effects of the two drugs were compared by using the overall symptom scores for all symptoms, BDA was shown to be significantly superior to flunisolide ($p<0.0005$).

The mean changes of the symptom scores of group I and group II were also recorded separately (Table 2).

This again shows significant symptomatic relief in both the BDA and the flunisolide groups.

The mean admission score for sneezing, stuffy nose and running

Table 1 Overall changes in mean symptom score in all patients using BDA and flunisolide (for one month each).

Symptom	Mean change from admission (mean ± S.D.)	
	Beclomethasone dipropionate group	Flunisolide group
Itching	− 1.18 ± 0.58***	− 0.44 ± 1.12*
Sneezing	− 1.56 ± 0.65***	− 0.8 ± 1.03***
Stuffy nose	− 0.93 ± 0.93***	− 0.87 ± 0.99***
Running nose	− 1.27 ± 0.69***	− 0.8 ± 1.03***
Total score	− 4.96 ± 1.99***	− 2.91 ± 3.34**

* = $P<0.05$; ** = $P<0.005$; *** = $P<0.0005$.
Calculated by using the paired-t test.

Table 2 Mean symptom scores on therapy. (mean ± S.D.)

Symptom	Group I Mean admission score	Group I Mean change from admission Period I (BDA)	Group I Mean change from admission Period II (flunisolide)	Group II Mean admission score	Group II Mean change from admission period I (flunisolide)	Group II Mean change from admission Period II (BDA)
Itching	1.76 ± 0.90*	− 1.39 ± 0.55***	− 0.42 ± 1.11*	1.08 ± 1.08	− 0.50 ± 0.996*	− 0.58 ± 0.67*
Sneezing	2.30 ± 0.68	− 1.64 ± 0.67***	− 0.76 ± 1.03***	1.92 ± 0.9	− 0.92 ± 0.95**	− 1.33 ± 0.79***
Stuffy nose	1.76 ± 0.83	− 1.00 ± 0.87***	− 0.79 ± 1.02***	2.08 ± 0.9	− 1.08 ± 0.95***	− 0.75 ± 0.98*
Running nose	2.00 ± 0.87	− 1.33 ± 0.60***	− 0.79 ± 1.02**	1.92 ± 1.08	− 0.83 ± 1.08*	− 1.08 ± 0.94***
Total scores	7.82 ± 1.70	− 5.40 ± 1.71***	− 2.76 ± 3.41***	7.00 ± 2.17	− 3.33 ± 3.05***	− 3.75 ± 2.60***

* = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.0005$
(using the paired-t test to compare the admission score with that of period I and period II)

nose between the two groups was not significantly different. Only the mean admission score for itching in group I was slightly more than that of group II; nevertheless, when the mean of the overall symptom scores was analysed, it showed no significant difference between the two groups.

Among the various investigations carried out during the trial, viz. absolute eosinophil count, eosinophils in the nasal smear, nasal provocation test and total serum IgE, the results were collected and recorded for three groups

1. The control group (= before commencing the treatment)
2. After using BDA for four weeks
3. After using flunisolide for four weeks

The result of the nasal provocative test was expressed by rating the nasal manifestations which occurred after the provocation on the 4-point scale as described earlier. The overall results of these investigations are summarised and shown in Table 3.

There were no significant changes in the absolute eosinophil count and the number of eosinophils in the nasal smear from the control after using both BDA and flunisolide. But the symptom scores rated after the nasal provocative test decreased significantly after each treatment. Total serum IgE increased in both treatment groups, but it reached the statistically significant level only in the flunisolide group.

Table 3 Various investigations accomplished at the beginning and at each follow-up visit.

	Control	BDA	Flunisolide
Abs. Eos. (mean ± S.E.)	528.46 ± 131.47	377.18 ± 49.74	290.40 ± 69.39
Eos. in nasal smear (mean ± S.D.)	5.11 ± 6.38	8.95 ± 16.78	3.71 ± 9.07
Nasal provocative test (mean ± S.D.)	3.53 ± 3.00	1.6 ± 1.50***	2.29 ± 2.25*
IgE (mean ± S.E.)	595.58 ± 62.43	663.55 ± 91.41	845.44 ± 214.07***

* = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.0005$
Calculated by paired-t test comparing the control and patients after using BDA and Flunisolide.

Three nasal swabs taken from every patient at the beginning and after each treatment period revealed some growth of bacteria and fungi but they did not differ significantly between each interval. No growth of Monilia was reported.

Most of the patients also used antihistamine tablets during the trial, but only in small amounts; they were comparable in both the BDA and flunisolide groups.

Three patients on BDA treatment and nine patients on flunisolide experienced some side-effects which were considered to be probably drug-related (see Table 4). Some patients reported more than one side-effect; however, only one patient with rash discontinued therapy after using flunisolide for three weeks, but we did not exclude this case from the study.

The assessments of the effectiveness of each kind of treatment by patients and physicians at the end of the trial are shown in Table 5.

The patients' and physicians' opinions were similar and both drugs showed a significant control of symptoms. There was no statistically significant difference between the two treatments ($p = 0.3305$ for patients and $p = 0.3394$ for physicians).

At the end of the study, the pa-

Table 4 Number of side-effects reported.

Side-effect	BDA	Flunisolide
Burning sensation	1	9
Nasal irritation	–	1
Nasal obstruction	1	–
Throat dryness	1	–
Headache	1	1
Dizziness	1	–
Insomnia + night mare	1	–
Rash	–	1
Total	6	12

Table 5 Comparison of effectiveness of drugs. (using the Chi-squared test)

Evaluated by	Very effective	Effective	Moderately effective	Not effective
Patients				
BDA	11	18	13	3
Flunisolide	9	6	13	17
(p = 0.3305)				
Physicians				
BDA	6	18	17	4
Flunisolide	4	11	12	18
(p = 0.3394)				

Very effective = 100% control of symptoms
Effective = 75% control of symptoms
Moderately effective = 50% control of symptoms
Not Effective = < 50% control of symptoms

Table 6 Preferences at end of study.

	BDA	Flunisolide	Same	None
Patients	35 (77.78%)	7 (15.56%)	1 (2.22%)	2 (4.44%)

tients expressed a preference for BDA (see Table 6).

DISCUSSION

In general, the overall symptom scores and the patient preference in our study have shown that BDA is superior to flunisolide. This is quite a different outcome than that of another comparative study where flunisolide was proved to be equally effective as BDA.[13] Some of our patients noted that both BDA and flunisolide were equally effective in controlling their nasal symptoms; however, they preferred the use of BDA. One possible reason for this high preference may be due to the different presentation of the two drugs. Flunisolide nasal spray is a mildly viscous aqueous solution of 0.025% flunisolide in a mixture of 20% propylene glycol and 15% polyethylene glycol delivered by a metered dose pump while beclomethasone dipropionate is a suspension propelled by fluorochlorohydrocarbon (Freon) delivered by a metered dose aerosol. The presentation of BDA and its vehicle may have been more acceptable for our patients. Among many side-effects reported by flunisolide users, a burning sensation in the nose was the most frequent. This is probably due to its vehicle; in the earlier studies which compared flunisolide with its vehicle control, this side-effect was also encountered. This vehicle provides an advantage over the mild discomfort encountered as it makes possible the administration of flunisolide via the intranasal spray without having to use halogenated hydrocarbon propellants.

Another complaint of a few patients who using flunisolide nasal spray was that the solution ran down the back of their throat and sometimes returned through their nostrils. However, this complaint is not considered a side-effect. It is interesting to note that epistaxis, which is one of the side-effects reported in a study of intranasal corticosteroids, in the Western Hemisphere, did not happen in our patients. This may be attributable to the climate of our country which is warm and humid rather than to the drug itself.

Our results confirmed the findings that both BDA and flunisolide are safe and effective treatments for patients with perennial rhinitis. In the group of patients who preferred the use of flunisolide nasal spray, there were some who really appreciated its effective control of their nasal symptoms. Furthermore, the twice-a-day dosage regimen of fluni-

solide is also more suitable for the patient than the four-times-a-day dosage of BDA.

Therefore, flunisolide was certainly valuable as an intranasal treatment in the group of perennial rhinitis patients who responded satisfactorily to its use. It was also valuable as an alternative in another group of perennial rhinitis patients who did not respond to BDA or who used to respond well but after long-term use, developed tolerance to BDA.

In a comparative study, flunisolide was shown to be significantly superior to sodium cromoglycate in the overall assessment of symptom control in hay fever patients.[23] Flunisolide aerosol was also found to be an effective and well-tolerated alternative to oral corticosteroids in the treatment of steroid-dependent asthma in both adults[24] and children.[25] In a study of a group of patients using BDA for asthma, their accompanying perennial rhinitis was substantially controlled by flunisolide nasal solution without significant effect on plasma cortisol levels and on the incidence of overgrowth of Candida.[26] Flunisolide used at the therapeutic dose for the treatment of perennial rhinitis for a period of three months was proven to exert no significant effect on the collagen content or the surface epithelium of the nasal mucosa. There was also no sign of atrophic rhinitis or any infective process induced by the drug.[27] Hence, flunisolide is another form of topical steroid which can be used alternatively or concurrently with BDA for controlling allergic symptoms of the airways with considerable effect and safety.

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REFERENCES

1. Mygind N. Local effect of intranasal beclomethasone dipropionate aerosol in hay fever. Br Med J 1973; 4:464-6.
2. Bunnag C, Vipulakom P, Pacharee P, Siriyananda C. Intranasal inhalation of beclomethasone dipropionate in the treatment of perennial rhinitis in adults. Ann Allergy 1980; 44:100-5.
3. Bloom FL, Cohan RH, Leifer KN, Spangler DL, Rhondes RB, Wittig HJ. Flunisolide aerosol in the treatment of perennial allergic rhinitis. Ann Allergy 1977; 38:408-51.
4. Schulz JI, Johnson JD, Freedman SO. Double-blind trial comparing flunisolide and placebo for the treatment of perennial rhinitis. Clin Allergy 1978; 8:313-20.
5. Horan JD, Johnson JD. Flunisolide nasal spray in the treatment of perennial rhinitis. CMAJ 1978; 119:334-8.
6. Sahay JN, Chatterjee SS, Engler C. Flunisolide – a new intranasal steroid for the treatment of allergic rhinitis. Clin Allergy 1979; 9:17-24.
7. Backhouse CI. Intranasal flunisolide in the treatment of allergic rhinitis in general practice. Curr Med Res Opin 1979; 6:14-9.
8. Saito Y. Treatment of perennial and seasonal allergic rhinitis with flunisolide nasal spray: An open trial and a multi – centre double-blind trial. Rhinology 1981; 1 (suppl.): 215-8.
9. Turkeltaub PC, Norman PS, Crepea S. Treatment of ragweed hay fever with an intranasal spray containing flunisolide, a new synthetic corticosteroid. J Allergy Clin Immunol 1976; 58:597-606.
10. Kammermeyer JK, Rajtora DW, Anuras J, Richerson HB. Clinical evaluation of intranasal topical flunisolide therapy in allergic rhinitis. J Allergy Clin Immunol 1977; 59:287-93.
11. Strem EL, Austrian S, Geller GR, Johnson JD, Crepea S. Flunisolide nasal spray for the treatment of children with seasonal allergic rhinitis. Ann Allergy 1978; 41:145-9.
12. Sarsfield JK, Thomson GE. Flunisolide nasal spray for perennial rhinitis in children. Br Med J 1979; 2:95-97.
13. Sahay JN, Chatterjee SS, Engler C. A comparative trial of flunisolide and beclomethasone dipropionate in the treatment of perennial allergic rhinitis. Clin Allergy 1980; 10:65-70.
14. Mygind N, Hansen I, Pedersen CB, Prytz S, Sorensen H. Intranasal beclomethasone dipropionate aerosol in allergic nasal diseases. Postgrad Med J 1975; 51 (suppl. 4):107-10.
15. Syntex Corporation, unpublished data.
16. Brown HM, Storey G, George WHS. Beclomethasone dipropionate: a new steroid aerosol for the treatment of allergic asthma. Br Med J 1972; 4:585-90.
17. Gibson GJ, Maberly DJ, Lal S, Ali MM, Butler AG. Double-blind, crossover trial comparing intranasal beclomethasone dipropionate and placebo in perennial rhinitis: Br Med J 1974; 4:503-4.
18. Prahl P, Wilken-Jensen K, Mygind N. Beclomethasone dipropionate aerosol in treatment of hay fever in children. Arch Dis Child 1975; 50:875-8.
19. Shore SC, Weinberg EG. Beclomethasone dipropionate aerosol in treatment of perennial allergic rhinitis in Children. Arch Dis Childh 1977; 52:486-8.
20. Choovoravech P, Preeyasombat C. Trial of beclomethasone dipropionate aerosol in the treatment of severe chronic asthma. Practitioner 1976; 220:639-42.
21. Harris DM, Martin L, Harrison C, Jack D. The effect of intranasal beclomethasone dipropionate on adrenal function. Clin Allergy 1977; 4:291-4.
22. Miyamoto T, Osawa N, Makino S, Hariuchi Y. Effect of beclomethasone dipropionate inhalation on adrenal function. Postgrad Med J 1975; 5:25-7.
23. Brown HM, Engler C, English JR. A comparative trial of flunisolide and sodium cromoglycate nasal sprays in the treatment of seasonal allergic rhinitis. Clin Allergy 1981; 11:169-73.
24. Slavin RG, Izu AE, Bernstein IL, Blumenthal MN, Bolin JF, Ouellette JJ, Reed CE, Oren J. Multicenter study of flunisolide aerosol in adult patients with steroid-dependent asthma. J Allergy Clin Immunol 1980; 66:379-85.
25. Orgel HA, Meltzer EO, Kemp JP. Flunisolide aerosol in treatment of steroid-dependent asthma in children. Ann Allergy 1983; 51:21-5.
26. Rusnak SL. Concurrent administration of flunisolide nasal solution with beclomethasone dipropionate bronchial aerosol in patients with both rhinitis and asthma. Ann Allergy 1981; 47:320-4.
27. Sahay JN, Ibrahim NBN, Chatterjee SS, Nassar WY, Lodge KV, Jones CW. Long-term study of flunisolide treatment in perennial rhinitis with special reference to nasal mucosal histology and morphology. Clin Allergy 1980; 10:451-7.

H-2 Compatibility Required for Tolerance Induction in Contact Sensitivity to DNFB in Allogeneic Bone Marrow Murine Chimeras*

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SUMMARY **Using irradiated bone marrow murine chimeras, we analysed the H-2 compatibility required for tolerance induction to the contact sensitiser DNFB (2, 4-dinitrofluorobenzene). In our previous reports, we showed that a previous injection of DNBS (2, 4-dinitrofluorobenzene sulfonic acid sodium salt) failed to induce tolerance to the relevant antigen (DNFB) in H-2-incompatible bone marrow chimeras, but it succeeded in H-2-compatible chimeras. In the present study, we employed a number of chimeras constructed from various combinations of marrow cells from B10 H-2 recombinant strains and AKR recipient mice to evaluate fine subregion compatibility at the H-2 complex required for the induction of tolerance. We found that total H-2 compatibility was required for tolerance induction in these chimeras.**

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Bone marrow transplantation is an important manoeuvre in the field of fundamental research on immunology[1-4] as well as in clinical applications.[5] This "cellular engineering" manoeuver[6] has been used with mice for the last decade; irradiated recipient mice have been reconstituted with bone marrow cells from either syngeneic, semi-allogeneic or allogeneic strains. In our laboratory, several immunological characteristics of bone marrow chimeric mice have been studied.[7-11] These characteristics include humoral immune response,[7-9] contact sensitivity,[7,10] cytotoxic T-lymphocyte (CTL) activity,[7] natural killer (NK) cell activity,[7] and granuloma formation to BCG cell-wall vaccine.[11]

The experimental system for the study of contact sensitivity in mice is well established.[12,13] Hypersensitivity, specific for a relevant hapten group, can easily be evoked following sensitisation with 2, 4-dinitrofluorobenzene (DNFB). On the other hand, when mice are injected intravenously with 2, 4-dinitrobenzene sulphonic acid sodium salt (DNBS), which shares antigenic moieties with DNFB, the mice will not show any manifestations of hypersensitivity following sensitisation with DNFB.[14] This phenomenon has been studied extensively as one of the experimental models for "immunological tolerance" and it is well established that suppressor T cells are responsible for the tolerance induction.[15]

We previously reported that H-2-incompatible bone marrow chimeras were unable to induce immunological tolerance to DNFB following intravenous injection with DNBS.[7,10] From these data, we postulated that some of the pathways involved in the induction of tolerance did not work in these animals as had been shown in the system of primary antibody response to sheep erythrocytes.[7,8] These pathways may include macrophage-T cell interaction, T-T cell interaction(s) and soluble factor(s)-T cell interaction(s); some of these interactions have been demonstrated to work under the control of the H-2 gene (H-2 restriction).[16-18] In the present study, we

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prepared a number of chimeras constructed from various combinations of marrow cells from B10 H-2-recombinant mice and AKR recipients to evaluate fine histocompatibility between donor and recipient animals required for the induction of tolerance to DNFB. The results strongly suggest that in this experimental system bone marrow recipients must share the total H-2 region with bone marrow donors in order to show immunological tolerance following intravenous injection with DNBS.

MATERIALS AND METHODS

Mice

Female B10. A(2R), B10. A(5R), B10. AKM and AKR were purchased from Jackson Laboratory, Bar Harbor, Maine, U.S.A. Female B10. A(3R), B10. A(4R) and B10. AQR were produced in our animal faculty. First, two strains were originated from the breeding pairs kindly previded by Dr. T. Hamaoka of Osaka University and the B10. AQR mice were provided by Dr. K. Okuda of Yokohama City University. Mice 8-12 weeks old were used as irradiated recipients; mice 7-10 weeks old, as bone marrow cell donors. All mice were maintained on sterilised water and a conventional diet *ad libitum*.

Bone marrow chimeras

Chimeric mice were prepared as described previously.[7,10,19] Briefly, recipient mice were irradiated with 880-900 R of X-radiation and injected with 25 x 10^6 bone marrow cells pretreated with monoclonal anti-Thy 1.1 (clone T11D7e, Olac Ltd., England) or anti-Thy 1.2 (clone F7D5, Olac). All chimeric mice were allowed to rest more than eight weeks prior to analyses for chimerism and contact sensitivity. (For simplicity, an irradiated AKR chimera reconstituted with B10.A(2R) cells is referred to herein as [B10.A(2R) → AKR]; other chimeras established with different combinations are referred to according to this nomenclature.)

Cytotoxic test

All chimeric mice were analysed for chimerism by the cytotoxic test as described earlier.[7] Thymocytes were incubated with anti-Thy 1.1, anti-Thy 1.2 or medium 199 followed by incubation with rabbit complement. The percentage of dead cells was evaluated by using the dye-exclusion method with 0.2% trypan blue; the cytotoxic index (C.I.) was determined according to the following formula:

$$\text{C.I.} = \frac{(\%\text{ of dead cells in experiment}) - (\%\text{ of dead cells in complement control})}{100 - (\%\text{ of dead cells in complement control})} \times 100$$

Proliferative response to mitogen

Spleen cells (5 x 10^5) were cultured with 1 μl/ml of phytohaemagglutinin (PHA) (Difco Laboratories, Detroit, U.S.A.), 5 μg/ml of Con A (Sigma Chemical Co., St. Louis, U.S.A.) or 25 μg/ml of *E. coli* LPS (Difco Laboratories) in a 96-well round-bottom plate (A/S Nunc, Denmark) in 200 μl of RPMI 1640 (Gibco Laboratories, Grand Island, N.Y., U.S.A.) supplemented with 10% foetal calf serum (Gibco Laboratories), 100 U/ml of penicillin and 100 μg/ml of streptomycin. The plate was cultured in a humidified 5% CO_2 atmosphere at 37°C for 72 hours with 0.5 μCi [methyl-^{3}H] thymidine (NET-027, New England Nuclear, Boston, U.S.A.) added during the final 20 hours. Cultures were harvested onto a glass filter and processed for liquid scintillation counting. Arithmetic means of triplicate cultures were presented in counts per minute (cpm).

Contact sensitivity and tolerance induction to DNFB

DNFB and DNBS were obtained from Wako-Junyaku Co., Tokyo. Sensitisation with DNFB and tolerance induction by DNBS were based on the method of Phanuphak *et al.*[14] The mice were sensitised by two daily paintings on the shaved abdomen using 25 μl of 0.5% DNFB in a 4:1 acetone:olive oil vehicle. Five days after the first painting, the mice were challenged with 20 μl of 0.2% DNFB on the right ear and with 20 μl of vehicle on the left ear as control. Twenty-four hours later, ear thickness was measured with a dial thickness gauge (Model G, Peacock C, Tokyo). The responsiveness of the mice to contact sensitisation was calculated by subtracting the thickness of the left ear from the thickness of the right ear and expressed in units of 10^{-2} mm. Tolerance to DNFB was induced by intravenous injection of DNBS (750 mg/kg) seven days before sensitisation with DNFB.

RESULTS

Chimerism by allogeneic marrow transplantation

All the chimeric mice were evaluated for chimerism when they were sacrificed for experimentation following 8-12 weeks of rest after irradiation. Thymocytes of the chimeric mice were estimated on

Table 1 Thy 1 phenotype of chimeric mice

Strain	Cytotoxic Index (%)	
	Thy 1.1	Thy 1.2
[B10. A(2R)→AKR] *	4	79
[B10. A(3R→AKR]	3	81
[B10. A(4R)→AKR]	8	76
[B10. A(5R)→AKR]	6	85
[B10.AKM→AKR]	7	74
[B10.AQR→AKR]	15	82
[AKR→AKR]	60	11
C57BL/6	0	81

*[B10. A(2R)→AKR] indicates irradiated ARK mice reconstituted with bone marrow cells of the B10. (22R) stain.

Thy 1 phenotype by means of cytotoxic test. As shown in Table 1, thymocytes obtained from AKR mice (Thy 1.1) reconstituted with B10 congeneic mice (Thy 1.2) showed a Thy 1.2 phenotype as did those from normal B6 mice. These data demonstrate that all mice were reconstituted with cells of the donor phenotype, thus were full chimeras.

Responses of spleen cells of chimeric mice to mitogens

Next, we observed proliferative responses to three kinds of mitogens (PHA, Con A, LPS) to evaluate the T cell and B cell reconstitution. Representative experiments comparing responses to these mitogens of spleen cells from six kinds of chimeras, [AKR→AKR] syngeneic marrow transplanted mice and normal B6 mice are shown in Table 2. The last group of B6 mice comprised three subgroups, which had been sensitised with DNFB or toleralised with DNBS or left untreated. As the table shows, all groups of chimeras responded at a level comparable to that of normal B6 mice, which result was consistent with previous observations on other chimeric mice.[7,10] Sensitisation with DNFB or desensitisation with DNBS had no influence on their responses.

Based on these results, it was assumed that all irradiated mice had been successfully reconstituted with bone marrow cells of donor origin and that the lymphoid cells of the mice were well developed and sufficiently mature to respond to the mitogens. These mice were then used for contact sensitivity experiments.

Contact sensitivity and tolerance induction to DNFB in chimeras

To investigate interactions of the immuno-competent cells and the host cells or cellular components, an analysis was carried out with regard to the capacity of the H-2-subregion-compatible chimeras to develop and express contact sensitivity to DNFB, using the ear swelling technique of Phanuphak *et al.*[14] As shown in previous papers,[7,10,20] in which H-2-incompatible allogeneic chimeras showed vigorous responses in contact sensitivity expression, a vigorous capacity to develop and express contact sensitivity was also present in the H-2-subregion-compatible chimeras (data not shown).

Analyses to evaluate the capacity to produce specific tolerance to DNFB by intravenous administration of DNBS before the sensitising application of DNFB gave a different result. Ear swelling data on these chimeric mice are presented in Table 3 together with the H-2 map of the donor strains. Underlines in the H-2 map indicate histocompatible regions between donor and recipient strains. As may be seen in the table, tolerance was successfully induced in syngeneic [AKR→AKR] mice. However,

Table 2 Proliferative responses to mitogens in chimeric mice.

Strain	Incorporation of ^{3}H-Thymidine (cpm)			
	Medium	PHA	Con A	LPS
[B10. A(2R)→AKR]*	2,634	9,879	28,174	20,034
[B10. A(3R)→AKR]	2,697	12,220	28,793	17,477
[B10. A(4R)→AKR]	2,459	21,132	31,138	19,843
[B10. A(5R)→AKR]	1,109	5,836	31,205	15,351
[B10. AKM→AKR]	1,289	4,455	32,891	13,730
[B10. AQR→AKR]	1,461	10,257	33,682	13,547
[AKR→AKR]	3,763	9,447	37,145	21,790
C57BL/6 (sensitised)	2,324	14,443	44,386	14,338
C57BL/6 (tolerant)	952	12,936	54,200	16,898
C57BL/6 (normal)	784	9,722	46,388	15,092

*See footnote in Table 1.

Table 3 Tolerance induction requiring total H-2 compatibility between donor and recipient mice.

Strain	H-2*							No.	DNBS	DNFB	Ear swelling (x 10^2 mm)
	K	A	B	J	E	C	D		iv+	sens+	mean ± s.d.
[B10. A(2R)→AKR]†	*k*	*k*	*k*	*k*	*k*	d	b	6	+	+	7.0 ± 3.0
[B10. A(3R)→AKR]	b	b	b	b	*k*	d	d	6	+	+	12.8 ± 3.5
[B10. A(4R)→AKR]	*k*	*k*	b	b	b	b	b	6	+	+	13.0 ± 4.1
[B10. A(5R)→AKR]	b	b	b	*k*	*k*	d	d	7	+	+	7.7 ± 5.3
[B10. AKM→AKR]	*k*	*k*	*k*	*k*	*k*	*k*	q	5	+	+	8.0 ± 1.6
[B10. AQR→AKR]	q	*k*	*k*	*k*	*k*	d	d	5	+	+	9.8 ± 4.1
[AKR→AKR]	*k*	*k*	*k*	*k*	*k*	*k*	*k*	4	+	+	2.0 ± 0.8
C57BL/6 (sensitised)								5	–	+	12.0 ± 2.5
C57BL/6 (tolerant)								5	+	+	1.8 ± 0.8
C57BL/6 (normal)								3	–	–	1.7 ± 0.6

† See footnote in Table 1.

* H-2 maps of bone marrow donor strain are presented. Underlined regions are histocompatible between donor and recipient (AKR, H-2^k).

+ Mice were injected with DNBS seven days before DNFB sensitisation.

neither group of chimeric mice reconstituted with B10 congenic strains showed a manifestation of tolerance to DNFB, even if recipient AKR mice shared almost all H-2 regions (except for the H-2D region) with the donor strain B10. AKM, [B10.AKM→AKR].

DISCUSSION

In our previous reports,[7,10] it was demonstrated that [B6→C3H] and [B6→AKR] H-2- incompatible chimeras developed and expressed contact sensitivity to DNFB. However, those mice appeared to be completely unable to develop the specific unresponsiveness to stimulation with DNFB by the intravenous administration of DNBS. By contrast, AKR mice reconstituted with H-2-compatible bone marrow cells from E^k mice (B6 congenic mice with H-2^k) showed successful induction of tolerance.[7] Since expression of tolerance to DNFB by the intravenous route is mediated by suppressor T cells,[12,13,15] it was suggested that the AKR recipient mice had to share the H-2 region with the donor strain for the suppressor T cells to inhibit the response.

In the present study, several groups of bone marrow chimeras were prepared to analyse the precise requirements of histocompatibility within the H-2 complex for tolerance-induction to DNFB. B10 congenic and B10. A intra-H-2 recombinant strains were introduced as bone marrow donors to establish such chimeras. These chimeric mice had contributed to the elucidation of fine genetic restriction in primary humoral response as described elsewhere.[8,21]

It was clearly demonstrated that tolerance was induced by DNBS only when the recipient mice were reconstituted with marrow cells from a syngeneic donor. In [B10. AKM→AKR] chimeric mice, where AKR recipients shared the greater part of the H-2 region (except for H-2D) with the B10. AKM donor, tolerance could not be demonstrated. This finding suggests that total H-2 identity between donor and recipient is required for tolerance induction. These results are in striking contrast to the findings regarding primary humoral responses to sheep erythrocytes,[8,9,21] where compatibility at the left one half of the H-2 region was shown to be sufficient to produce antibody. It seems that the apparent discrepancy is due to the difference in cell types involved in these experimental systems.

Suppressor T cell (Ts) mechanisms exerting "in contact" sensitivity to DNFB have been intensively investigated using several experimental systems, viz. Ts induced by DNBS,[14-17] Ts induced by DNP-modified spleen cells (reviewed in references 12 and 13) or Ts induced by TNBS.[18] However, there is a marked heterogeneity with regard to the nature of Ts involved in these systems, i.e. intravenous administration of DNBS induces Ts functioning in the afferent phase and TNBS activates efferent phase-acting Ts.[22] In the former system, Moorhead[15,16,23] proposed that the suppression of the sensitivity was mediated by a factor (soluble suppressor factor, SSF). Following activation of SSF-producing cells (Ts?) by DNBS, these cells release SSF after stimulation by DNFB painting. Thereafter, SSF, with H-2 antigenic moiety and binding capacity to DNP, interacts with DNFB-immune T cells resulting in a failure of effector T cells develop. For the interaction between the factor and the immune T cells, compatibility at the H-2K and/or H-2D region is required.

On acceptance of this illustration, our findings suggest that the chimeric mice in the present study carried some defects in these interactions. It seems to us that compatibility at the H-2K and H-2D regions between donor and recipient strains is required for the interactions to occur efficiently. However, the suppressor T-cell mechanism is made up of several steps at different stages. Furthermore, when irradiation bone marrow chimeras are employed to analyse immune functions, sometimes wide variations in responsiveness are observed; these are probably influenced by the differences in the strain combinations to establish the chimeras and in the nature of antigens used in the assay systems.[20,24] Further analyses remain to be performed.

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REFERENCES

1. Bevan M. In a radiation chimera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. Nature (Lond.) 1977; 269:417-8.
2. Katz DH, Skidmore BJ, Katz LR, Bogowitz CA. Adaptive differentiation of murine lymphocytes. I. Both T and B lymphocytes differentiating in F1 parental chimeras manifest preferential cooperative activity for partner lymphocytes derived from the same parental type corresponding to the chimeric host. J Exp Med 1978; 148:727-45.
3. Kraal G, Van Der Hirst B, Boden D. Functional and morphological recovery of the T-cell compartment in lethally irradiated and reconstituted mice. Immunology 1979; 37:195-201.
4. Gorczynski RM, Macrae S. Differentiation of functionally active mouse T lymphocytes from functionally inactive bone marrow precursors. IV. Recovery of T-cell function from bone marrow precursors in a histoincompatible environment. Immunology 1979; 38:13-22.
5. Good RA, Bach FH. Bone marrow and thymus transplantation: Cellular engineering to correct primary immunodeficiency. Clin Immunobiol 1974; 2:63-114.
6. Good RA. Cellular engineering. Clin Bull 1977; 7:33-9.

7. Onoé K, Fernandes G, Good RA. Humoral and cell-mediated immune responses in fully allogeneic bone marrow chimera in mice. J Exp Med 1980; 151:115-32.
8. Onoé K, Yasumizu R, Oh-ishi T, Kakinuma M, Good RA, Morikawa K. Restricted antibody formation to sheep erythrocytes of allogeneic bone marrow chimeras histoincompatible at the K and of the H-2 complex. J Exp Med 1981; 153:1009-14.
9. Iwabuchi K, Onoé K, Ogasawara M, Yasumizu R, Morikawa K. Primary and secondary antibody responses to sheep erythrocytes of allogeneic bone marrow chimeras histocompatible at the left or right one-half of the H-2 complex. Hokkaido J Med Sci 1983; 58:232-7.
10. Yasumizu R, Onoé K, Ogasawara M, Iwabuchi K, Geng L, Morikawa K. Contact sensitivity in fully allogeneic bone marrow chimera in mice, [B6→C3H]. Asian Pacific J Allerg Immun 1984; 2:61-6.
11. Kakinuma M, Onoé K, Yasumizu R, Yamamoto K. Strain differences in lung granuloma formation in response to a BCG cell-wall vaccine in mice. Failure of antigen presentation by low-responder macrophages. Immunology 1983; 50:423-31.
12. Claman HN, Miller SD, Sy M-S, Moorhead JW. Suppressive mechanisms involving sensitization and tolerance in contact allergy. Immunol Rev 1980; 50:105-32.
13. Claman HN, Miller SD, Conlon PJ, Moorhead JW. Control of experimental contact sensitivity. Adv Immunol 1980; 30:121-57.
14. Phanuphak P, Moorhead JW, Claman HN. Tolerance and contact sensitivity to DNFB in mice. I. *In vivo* detection by ear swelling and correlation with *in vitro* cell stimulation. J Immunol 1974; 112:115-23.
15. Phanuphak P, Moorhead JW, Claman HN. Tolerance and contact sensitivity to DNFB in mice. III. Transfer of tolerance with "suppressor T cells". J Immunol 1974; 113:1230-6.
16. Moorhead JW. Soluble factors in tolerance and contact sensitivity to DNFB in mice. II. Genetic requirements for suppression of contact sensitivity by soluble suppressor factor. J Immunol 1977; 119: 1773-7.
17. Moorhead JW. Soluble factors in tolerance and contact sensitivity to DNFB in mice. III. Histocompatibility antigens associated with the hapten dinitrophenol serve as target molecules on 2, 4-dinitrofluorobenzene-immune T cells for soluble suppressor factor. J Exp Med 1983; 150: 1432-47.
18. Ptak W, Gershon RK, Flood PM. Mechanisms of suppression in the transfer of contact sensitivity. Analysis of an I-J$^+$ molecule required for Ly 2 suppressor cell activity. J Exp Med 1983; 158:1822-35.
19. Onoé K, Yasumizu R, Oh-ishi T, et al. Specific elimination of the T lineage cells: Effect of *in vitro* treatment with anti-Thy 1 serum without complement on the adoptive cell transfer system. J Immunol Methods 1982; 49:315-22.
20. Yasumizu R, Onoé K, Iwabuchi K, Ogasawara M, Geng L, Morikawa K. Analysis of contact sensitivity to 2, 4-dinitrofluobenzene (DNFB) in allogeneic bone marrow chmera in mice. Immunology (in press).
21. Onoé K, Yasumizu R, Geng L, et al. Analysis of Ia restriction specificity of helper T cells in H-2 subregion compatible bone marrow chimera in mice. Immunobiol (in press).
22. Asherson GL, Zembala M, Thomas WR, Perera MACC. Suppressor cells and the handling of antigen. Immunol Rev 1980; 50:3-45.
23. Moorhead JW. Soluble factor in tolerance and contact sensitivity to 2, 4-dinitrofluorobenzene in mice. I. Suppression of contact sensitivity by soluble suppressor factor released *in vitro* by lymph node cell populations containing specific suppressor cells. J Immunol 1977; 119:315-21.
24. Zinkernagel RM, Sado T, Althage A, Kamisaku H. Antiviral immune response of allogeneic irradiation bone marrow chimeras: cytotoxic T cell responsiveness depends upon H-2 combination and infectious agent. Eur J Immunol 1984; 14:14-23.

Pulmonary Function in Symptom-free Asthmatic Children*

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SUMMARY **Asthmatic children may be susceptible to more severe chronic airway obstruction during symptom-free periods if pulmonary function impairment is permanent and proper management is not given. This study was undertaken to observe whether the pulmonary function of such children differs from that of normal children so that preventive measures may be takan. Using a Godart Pulmotest Spirometer, the forced vital capacity (FVC), forced expiratory volume (FEV) in the first second ($FEV_{1.0}$) and mid-maximal expiratory flow rate (MMEFR) were measured. Clinical evaluation was also performed at the same 7 ± 1-week interval for a period of one year.**

Statistical analysis revealed 1) pulmonary function abnormalities in symptom-free asthmatic children, and 2) significant improvement after the administration of bronchodilator. Early detection of these abnormalities and the provision of a bronchodilator could, perhaps, prevent the further development of more severe chronic airway obstruction.

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Airway obstruction is one of the main features of bronchial asthma. Therefore, its accurate assessment is important with regard to clinical diagnosis and management. Some asthmatic children were considered to be in remission when clinical recovery from asthma was apparent.[1] However, increased bronchial smooth muscle tone may remain present, which probably renders such children susceptible to more severe chronic airway obstruction in the fature.[2]

This study was undertaken to observe whether the pulmonary function of such children differs from that of normal children so that preventive measures may be provided.[3]

MATERIALS AND METHODS

Subjects

Pulmonary function was measured in 24 children aged 6-15 years who had been followed up continuously in a paediatric allergy clinic for over one year. They also fulfilled the following criteria: 1) history of bronchial asthma for over three years; 2) symptom-free period of more than two weeks and 3) not having received antiasthmatic treatment (i.e., bronchodilator, steroids, hyposensitisation, etc.) during the previous two weeks.

Methods

Complete histories, physical examinations and parental consent were obtained for all children. They had been trained to perform the pulmonary function test in advance. Using a Godart Pulmotest Spirometer, the following pulmonary function parameters were initially measured in all children: forced vital capacity (FVC), forced expiratory volume in the first second ($FEV_{1.0}$) and maximal mid-expiratory flow rate (MMEFR).[4] The children were classified into the following groups (Table 1):

Group I: Patients with normal pulmonary function test.

Group II: Patients with abnormal pulmonary function test, having at least one or more of the pulmonary function parameters that registered less than 70 per cent of the predicted mean value.

Group III: Patients with abnormal pulmonary function test who were

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Table 1 Age distributions among the three groups of subjects

Group	Number (n)	Age (years)		
		Range	Mean	sd
I	8	8-15	10.9	2.59
II	8	7-15	10.8	2.71
III	8	7.5-15	12.4	2.41

Table 2 Mean ($\bar{x}$) and standard deviation (sd) of pulmo-parameters of the first measurement (*time period 1*)

Group		FVC (ml)	$FEV_{1.0}$ (ml)	MMEFR (l/min)
I	Actual	1,903.02	1,752.25	144.77
		±687.99	±674.53	±44.12
	Predicted	2,121.00	1,799.82	140.24
		±707.96	±711.90	±40.08
	Actual/Predicted	0.89	0.98	1.08
		±0.04	±0.06	±0.08
II	Actual	1,620.98	1,422.55	111.48
		±531.24	±520.12	±31.50
	Predicted	2,329.21	2,050.35	153.23
		±710.88	±726.15	±42.76
	Actual/Predicted	0.69	0.69	0.72
		±0.05	±0.02	±0.02
III	Actual	1,885.12	1,697.85	114.90
		±462.16	±398.78	±26.70
	Predicted	2,601.04	2,396.44	169.08
		±636.36	±541.95	±35.12
	Actual/Predicted	0.72	0.70	0.68
		±0.02	±0.02	±0.06

administered an oral bronchodilator *on a continuous basis* (treated group).

Group I and II was given the oral bronchodilator only *on an intermittent basis* (non treated groups) and the pulmonary function tests were performed after discontinuation of the medication for at least two weeks.

The study began eight weeks after the selection and grouping of the children. The pulmonary function parameters (FVC, $FEV_{1.0}$ and MMEFR) were measured at the same time (around 9 a.m.) for all children (Table 2). All children were retested and closely followed up clinically every 7±1 weeks for a period of one year (Table 3). All values have been corrected for barometric pressure and temperature saturation (BPTS). Each series of measurements lasted about 15 minutes. The children rested for 15 minutes before each test. Measurements were taken while the patients were sitting. The most uniform reading of the three attempts was accepted for each test. For the groups undergoing treatment, the first measurement was performed on the first day of the treatment (Table 2).

Statistical analysis was done using Barlett's multiple test and analysis of variance (ANOVA) using an orthogonal factorial design[5] with patient groups and the period of measurement as main variables. A rejection criterion of 0.01 was used for judging significance. The pulmo-parameters for normal Thai children using the standard spirometer (Godart Pulmotest Spirometer) had not yet been established; thus the mean values and normal ranges presented by Polgar and Promadhat[6] were used.

RESULTS

Differences in the age of the children could be expected to account for differences in height which would affect pulmonary function parameters. A base-line analysis of age among the three groups of subjects (shown in Table 1) reveals no significant differences with regard to age statistics (Barlett's multiple t-test; $p > 0.30$).

Table 3 gives the results for FVC, $FEV_{1.0}$ and MMEFR respectively for the three groups. Analysis of variance (Tables 4-7) shows significant differences among the three groups, for all pulmo- parameters ($p<0.001$), but no significant differences with regard to time periods ($p = 0.805$, 0.489 and 0.607 respectively). Although the effect of the group-time interaction is controlled, the difference in FVC, $FEV_{1.0}$ and MMEFR among groups is significant ($p<0.001$).

Tables 7 to 9 show ANOVA data for FVC, $FEV_{1.0}$ and MMEFR for the treated and non-treated abnormal groups. Only the FVC parameter shows a significant difference between the two groups ($p = 0.009$).

DISCUSSION

The importance of early diagnosis of airway obstruction in asthma is not yet clear. So far, further information is required about its natural history and whether it may be possible to use early detection and management to prevent the development of more severe chronic airway obstruction in the future. Several authors have studied with variable results[1,2] asthmatic children

Table 3 Mean ($\bar{x}$) and standard deviation (sd) of pulmo-parameter (FVC, $FEV_{1.0}$ and MMEFR) of the three groups of asthmatic children expressed as the ratio of actual value to the predicted value on the basis of height

FVC

Group		Test period* 1	2	3	4	5	6
I							
Actual/Predicted	$\bar{x}$	0.9245	0.9461	0.8720	0.9047	0.9249	0.8776
	sd	0.724	0.0857	0.0863	0.0937	0.0772	0.0824
II							
Actual/Predicted	$\bar{x}$	0.7622	0.8230	0.8034	0.8038	0.7757	0.7440
	sd	0.1178	0.1538	0.1033	0.1024	0.1408	0.1520
III							
Actual/Predicted	$\bar{x}$	0.8171	0.8430	0.8365	0.8918	0.8520	0.8908
	sd	0.1137	0.1089	0.1264	0.1120	0.1523	0.1333

$FEV_{1.0}$

Group		Test period* 1	2	3	4	5	6
I							
Actual/Predicted	$\bar{x}$	1.0375	1.0811	0.9599	0.9943	0.9868	0.8943
	sd	0.0730	0.1610	0.0628	0.1044	0.0878	0.1164
II							
Actual/Predicted	$\bar{x}$	0.8114	0.8392	0.8146	0.8029	0.7819	0.7421
	sd	0.1254	0.1143	0.0970	0.0998	0.1287	0.1436
III							
Actual/Predicted	$\bar{x}$	0.7530	0.8091	0.7908	0.8222	0.8006	0.8754
	sd	0.1436	0.1232	0.1676	0.1465	0.1944	0.1247

MMEFR

Group		Test period* 1	2	3	4	5	6
I							
Actual/Predicted	$\bar{x}$	0.9245	0.9461	0.8720	0.9047	0.9249	0.8776
	sd	0.0724	0.0857	0.0863	0.0937	0.0772	0.0824
II							
Actual/Predicted	$\bar{x}$	0.7622	0.8230	0.8034	0.8038	0.7757	0.7440
	sd	0.1178	0.1538	0.1033	0.1024	0.1408	0.1520
III							
Actual/Predicted	$\bar{x}$	0.8171	0.8430	0.8365	0.8918	0.8520	0.8908
	sd	0.1137	0.1089	0.1264	0.1120	0.1523	0.1333

*At 6-8 weeks interval. Period 1 = the day the treatment was started, eight weeks after the initial measurements.

during symptom-free periods. Most of the studies showed that the majority of asthmatic children may show some slight abnormalities, particularly in their airway resistance, thus indicating a minimal and subclinical airway obstruction. In our study, we found abnormalities of pulmonary function in 16 out of 24 symptom-free children. This finding is in accordance with that reported by McFadden *et al*[7] who carried out their investigation among American children.

Our study further revealed that although asthmatic symptoms in children may disappear for a period of time, airway obstruction may remain. In the past, such airway blockage was not recognised because most tests for pulmonary functions were performed using conventional measurements such as peak flow rate and force expiratory volume in the first second. Tables 8 and 9 of our study substantiate this opinion. These tests primarily reflect changes in the large airway,[8] but they are not sensitive enough to detect abnormalities in the small airways.[8] Woolcock *et al*[9] and Heckscher *et al*[10] have shown that abnormalities of the small airways cause increased residual volume, mismatching of ventilation and perfusion, and abnormal frequency dependence of dynamic compliance. It is these silent changes in the periphery of the lung that permit abnormalities of gas exchange and lung mechanics to continue into the symptom-free period.[7,11,12] Unless sophisticated measures of the dynamics of small airways are made, the finding of $FEV_{1.0}$ or peak flow rate may encourage a false sense of security, i.e., that small as well as large airways may have returned to normal.[9,13]

The residual abnormalities may cause no symptoms at rest, but because of their persistence, they may predispose patients to future attacks.[14-16] This finding leads us to conclude that an oral bronchodilator should be given to symptom-free patients who have an abnormal

Table 4 Analysis of variance for forced vital capacity (FVC) among the three groups

Source of variation	SS	df	MS	F	p-value
Among groups	0.365	2	0.183	13.841	0.001
Among times	0.030	5	0.006	0.461	0.805
Interaction (groups vs. times)	0.076	10	0.008	0.573	0.834
Residual (error)	1.662	126	0.013	–	–
Total	2.133	143	0.015	–	–

Table 5 Analysis of variance for $FEV_{1.0}$ among the three groups

Source of variation	SS	df	MS	F	p-value
Among groups	1.142	2	0.571	30.330	0.001
Among times	0.072	5	0.014	0.891	0.489
Interaction (groups vs. times)	0.202	10	0.020	1.251	0.266
Residual (error)	2.037	126	0.016	–	–
Total	3.453	143	0.024	–	–

Table 6 Analysis of variance for MMEFR among the three groups

Source of variation	SS	df	MS	F	p-value
Among groups	3.553	2	1.776	28.524	0.001
Among times	0.225	5	0.045	0.723	0.607
Interaction (groups vs. times)	0.790	10	0.079	1.269	0.255
Residual (error)	7.847	126	0.062	–	–
Total	12.356	143	0.086	–	–

Table 7 Analysis of variance for FVC between the treated (Group III) and non-treated (Group II) abnormal groups

Source of variation	SS	df	MS	F	p-value
Between groups	0.117	1	0.117	7.169	0.009
Between times	0.031	5	0.006	0.374	0.865
Interaction (groups vs. times)	0.041	5	0.008	0.507	0.770
Residual (error)	1.371	84	0.016	–	–
Total	1.560	95	0.016	–	–

pulmonary function test. Goldstein *et al*[3] recommended the regular use of inhaled sympathomimetics and an oral theophylline preparation for those symptom-free patients whose history suggests that they are susceptible to acute exacerbation. Such patients commonly experienced an improved sense of well-being, increased exercise tolerance and a decrease in the frequency and severity of their acute episodes. Significant improvement of pulmonary function could be demonstrated in these patients after bronchodilator therapy judging by the improvement of the forced vital capacity,[17] which probably reflected the relief of peripheral airway obstruction and could be detected in the lower range of vital capacity.[18]

The pulmonary function tests which have been used in this study may not give a complete picture of lung function, but they do give a reasonable guide to the state of air movement. Most pulmonary diseases in childhood alter this aspect of respiration, so in practice these relatively simple bedside tests have been found to be valuable.[16] This is particularly true for long-term conditions such as asthma, for which they may give the only objective guide to the progress of the disease and the efficacy of treatment. These pulmonary function tests should be incorporated in the diagnosis and treatment of many types of respiratory illnesses. Asthmatic children should be checked regularly by their physicians, and they and their parents should be given guidance on the treatment of symptoms. They should be instructed in preventive measures and the promotion of health, i.e. breathing exercises, chest rehabilitation, etc. Thus, early detection of airway obstruction, although by simple but efficient pulmonary function tests, will no doubt provide advance warning which is helpful in preventing the development of more severe chronic airway obstruction in the future.

Table 8 Analysis of variance for $FEV_{1.0}$ between the treated and non-treated abnormal groups

Source of variation	SS	df	MS	F	p-value
Among groups	0.002	1	0.002	0.124	0.726
Among times	0.018	5	0.004	0.195	0.963
Interaction (groups vs. times)	0.901	5	0.018	0.977	0.437
Residual (error)	1.568	84	0.019	–	–
Total	1.680	95	0.018	–	–

Table 9 Analysis of variance for MMEFR between the treated and non-treated abnormal groups

Source of variation	SS	df	MS	F	p-value
Among groups	0.029	1	0.029	0.482	0.489
Among times	0.110	5	0.022	0.363	0.873
Interaction (groups vs. times)	0.692	5	0.138	1.219	0.054
Residual (error)	5.101	84	0.061	–	–
Total	5.915	95	0.062	–	–

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REFERENCES

1. Beale HD, Fowler WS, Comroe JH. Pulmonary function studies in 20 asthmatic patients in the symptom-free interval. J Allergy 1952; 23:1-10.
2. Orzalesi MM, Cook CH, Hart MC, Pulmonary function in symptom-free asthmatic patients. Acta Paediat 1964; 53:401-7.
3. Goldstein RS, Slutsky AS, Rebuck AS. Severe asthma: prevention is better than cure. Pract Therapeutics 1978; 16:256-67.
4. Dugdale AE, Mocri M. Normal values of forced vital capacity (FVC), forced expiratory volume ($FEV_{1.0}$), and peak flow rate (PFR) in children. Arch Dis Childh 1968; 43:229-34.
5. Dixon WJ, Massey FJ. Introduction to statistical analysis. 3rd Ed. New York: McGraw-Hill 1969:283-321.
6. Polgar G, Promadhat V. Pulmonary function testing in children. Techniques and standard. Philadelphia: W.B. Saunders 1973:255-6.
7. McFadden ER, Kiser R, DeGroot WJ. Acute bronchial asthma: relations between clinical and physiologic manifestations. New Engl J Med 1973; 288:221-5.
8. Colp CR. Interpretation of pulmonary function tests. (Editorials). Chest 1979; 76:377-8.
9. Woolcock AJ, Vincent NJ, Macklem PT. Frequency dependence of compliance as a test for obstruction in the small airways. J Clin Invest 1969; 48:1097-106.
10. Heckscher T, Bass H, Oriol A, Rose B, Anthonisen NR, Bates DV. Regional lung function in patients with bronchial asthma. J Clin Invest 1968; 47:1063-70.
11. Cade JF, Pain MCF. Pulmonary function during clinical remission of asthma. How reversible is asthma? Aus NJJ Med 1973; 3: 545-51.
12. Kerrebyn KF, Fioole AC, Bentveld RDW. Lung function in asthmatic children after a year or more without symptoms or treatment. Brit Med J 1978; 1:886-8.
13. Macklem PT, Mead J. Resistance of central and peripheral airways measured by a retrograde catheter. J Appl Physiol 1967; 22:395-401.
14. Rees HA, Millar JS, Donald KW. A study of the clinical course and arterial blood gas tensions of patients in status asthmaticus. Quart J Med 1968; 148:541-61.
15. McNeill RS, Nairn JR, Millar JS, Ingram CB. Excrecised-induced asthma. Quart J Med 1966; 137:55-67.
16. Brewis RAL. Practical pulmonary function tests. Practitioner 1977; 219:681-91.
17. Souhrada JF, Buckley JM. Pulmonary function testing in asthmatic children. Pediat Clin N Amer 1976; 23:249-78.
18. Cochrane GM, Benatar SR, Davis J, Collins JV, Clark TJH. Correlation between tests of small airway function. Thorax 1974; 28:172-8.

Isolation and Characterisation of C_1q from Human Serum*

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SUMMARY **The subcomponent C_1q, the first component of complement, was purified to homogeneity from human serum by affinity chromatography. The serum was euglobulin-precipitated at an alkaline pH; the reconstituted precipitate was chromatographed in rabbit anti-human C_1q covalently coupled to cyanogen-bromide-activated Sepharose-4B and also to Sepharose rabbit anti-normal human serum (C_1q depleted), C_1q was eluted by 1 M NaCl in the former while the flow-through of the latter was C_1q which was rechromatographed on a goat anti-human IgG Sepharose-4B column for removal of contaminating IgG.**

Final yields of C_1q ranged from 40 per cent to 70 per cent with a 420-to 490-fold purification of protein based on recovery of haemolytic activity. These preparations were free of contaminating serum proteins as judged by PAGE, SDS-PAGE and immuno-chemical criteria. However, the final C_1q preparation might have been contaminated with undetected C_1q inhibitor.

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Human C_1q is an unusual protein consisting of six triplet-stranded sub-units, each with a globular head that binds immunoglobulin and a collagen-like fibrillar tail that reacts with C_1r and C_1s.[1] It is of considerable interest because of its biological importance in initiating complement activation, the physical characterisation of C_1q, its interaction with C_1r and C_1s and with immunoglobulin. Also, C_1q has been used for the detection of circulating immune complexes in a wide variety of diseases[2,3] because it has the advantage of being a natural recognition protein for immune complexes by the classical complement system. For such studies, highly purified and biologically active C_1q is an essential prerequisite.

Various methods of C_1q isolation and purification utilise one or more of its chemical or biological properties. These include its minimal solubility in low ionic strength buffer,[4,5] reversible binding to heat-aggregated IgG,[6,8] precipitation with DNA[7] and reversible binding to solid phase human gammaglobulin with gel permeation and/or ion exchange chromatography.[8-12] In this communication, a rapid reproducible affinity column chromatography procedure for isolation of highly purified C1q is described.

MATERIALS AND METHODS

Serum: Human volunteers were bled; clotting proceeded at 37°C for two to three hours. The serum was obtained by wringing the clot and by centrifugation.

Reagents: Cyanogen bromide (CNBr)-activated Sepharose-4B and concanavalin-A were procured from Pharmacia Fine Chemicals, Uppsala, Sweden and Nobel Agar from Difco Laboratories, Detroit, MI, U.S.A. Acrylamide, N, N[1] methylene bis-acrylamide, Coomassie brilliant blue, sodium dodecyl sulphate (SDS), dithiothreitol (DTT), iodoacetamide, ethylene diamine tetra-acetic acid (EDTA), and methyl-D-glucopyranoside (MGP) were purchased from Sigma Chemicals, St. Louis, MO, U.S.A. Ammonium persulphate and N,N,N,N-tetramethylene diamine (TEMED) were purchased from Eastman, Rochester, N.Y., U.S.A. Antisera to IgG, IgM and IgA were produced locally and anti-C_1q was obtained from Cappel Laboratories, Malvern, PA, U.S.A.

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Immunoadsorbents

Rabbit anti-human C_1q RAH C_1q) was coupled to Sepharose-4B, 6g of CNBr-activated Sepharose-4B was washed in a Buchner funnel with 2 litres of 1 mM HCl followed by 250 ml of coupling buffer (carbonate-bicarbonate buffer, pH 8.9). Monospecific anti C_1q [2 ml (14.2 mg/ml)] was added and stirred gently for two hours at 37°C and subsequently washed with 0.1 M glycine-HCl, pH 2.5, followed by 0.01 M of phosphate buffered saline (PBS, pH 7.5). A protein determination was done on the filtrate and the amount of protein coupled was calculated to be 11-13 mg/ml Sepharose 4B.

Human IgG was purified by precipitation from sera with a 50 per cent final concentration of the ammonium sulfate and dialysed against PBS. The dialysate was centrifuged and applied to a DEAE cellulose column (2x20 cm) pre-equillibrated in PBS. Elution was carried out by increasing the salt concentration (0.005-0.2 M). Fractions containing IgG were pooled, concentrated and dialysed. Human IgG (HIgG) was coupled to CNBr-activated Sepharose-4B as described above and the amount of protein coupled was calculated to be 15-20 mg IgG/ml of Sepharose-4B. Fresh, pooled, normal human serum was made 10 mM in EDTA and applied to the HIgG Sepharose-4B column. The flow-through fractions were pooled and employed as C_1q-depleted (C_1qD) normal human serum (NHS).

Goat anti-human IgG (GAH IgG) and rabbit anti-NHS (C_1q-depleted) (RANHS) were prepared by immunising subjects with five subcutaneous injections of pure IgG and NHS (300-400 μg each) emulsified in complete Freund's adjuvant at weekly intervals. Two booster doses in incomplete Freund's adjuvant were given thereafter. Serum was obtained one week after the final injection and precipitated with 33% cold saturated ammonium sulphate at 4°C. After equillibrating the mixture for 30 minutes, the precipitate was collected by centrifugation (4,000 rpm, 10 min.), dissolved in PBS, exhaustively dialysed against PBS and coupled to CNBr-activated Sepharose-4B as described above.

The concanavalin A (Con A) Sepharose-4B column was equillibrated with 0.01 M veronal buffer, pH 8.0, containing 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM $MgCl_2$ (Con A buffer). Purified protein was applied and the column run at 22°C with a flow rate of 5 ml/hr; 1 ml fractions were collected. After the first peak, the solution was made at 10 per cent strength with alpha-methyl-D-glucopyranoside and bound protein was eluted.

Isolation procedure

Serum was titrated to pH 8.7-8.9, made 5 mM with EDTA and dialysed against 10 mM ethylene diamine-HCl, pH 8.8, for 20 hours in a ratio of 20:1 (buffer/serum). The mildly tubid solution was centrifuged at 4,000 g for 40 minutes to obtain C_1q-rich euglobulin precipitate and dissolved in 0.5 M NaCl containing 0.002 M EDTA, pH 7.4. Delipidification was carried out by centrifuging at 30,000 g for 90 minutes.

The $RAHC_1q$ and RANHS columns were washed prior to use with 0.1 M glycine-HCl, pH 2.5, followed by coupling buffer and finally with PBS containing 0.01 M EDTA. Euglobulin precipitate was applied and the column kept at 4°C overnight. $RAHC_1q$ was washed with 0.15 M NaCl buffer at a flow rate of 40 ml/hr until the absorbance at 280 nm of the flow-through material was less than 0.01. Elution of bound C_1q was carried out with 1 M NaCl in PBS containing 0.05 M EDTA (pH 7.5); fractions were concentrated and dialysed against PBS.

From the RAHNS column, the C_1q was in flow-through fractions, the bound material was eluted with 40% ethylene glycol. Fractions containing C_1q were rechromatographed on an RAH IgG Sepharose column to get rid of contaminating IgG. The RAH IgG column was prewashed with 0.01 M PBS containing 1.0 M NaCl, 0.05 M EDTA (pH 7.5).

Characterisation of C_1q

Immunoelectrophroesis (IEP) was carried out as described by Williams[13] using 1.5% agar in barbital buffer, pH 8.6. Electrophoresis was performed for six hours under a constant current of 5-10 mA per immunoframe. IgG, IgA and IgM were characterised in various fractions by Ouchterlony double diffusion[14] and quantified by radial immuno diffusion[15] in 1.5% agarose containing 0.01 M PO_4, 1.0 M NaCl (pH 7.5) and specific antibody. C_1q was detected by rocket immunoelectrophoresis as described by Laurell[16] and concentration was determined spectrophotometrically[17] using E_{280} 1% of 0.682. Protein concentration was determined by Folin phenol reagent[18] using bovine serum albumin standard.

C_1q haemolytic assay

The haemolytic activity of C_1q was assayed according to Kolb *et al.*[8] 20μl of appropriate dilution of the sample were added to a mixture containing 300 μl of GVB (isotonic veronal buffered saline containing 0.15 M $CaCl_2$, 0.5 mM $MgCl_2$ and 0.1% gelatin), 40 μl of C_1q-depleted serum (20 μl of 1 M $CaCl_2$ and 1 M $MgCl_2$ stock ml C_1qD) and 200 μl of EA ($5x10^7$/ml). The tubes were incubated at 37°C for 45 minutes at which time 1 ml of ice-cold GVB was added. After centrifugation, the OD of the supernatant at 412 nm was determined. The effective molecules were calculated according to Borsos and coworkers[19]

Polyacrylamide gel electrophoresis

PAGE was conducted using 7.5% gel in 0.1 M tris glycine buffer as described by Davis.[20] SDS–PAGE

Table 1. Purification of C_1q from human serum.

Fraction	Total protein	Total activity units*	Sp. activity units/mg protein	Yield	Purification (fold)	C_1q	Sp. activity mg C_1q/mg protein	Purification (fold)
Normal human serum	1,448	24,504	16.9	100.00		.5568	.0004	–
Euglobulin ppt	7.6	18,044	2,374.2	73.60	140	.5136	.0676	169
RAH C_1q column	0.031	16,935	8,338.2	69.10	492	.3867	.1904	476
RANHS column	2.025	14,657	7,238.0	41.00	427	.3395	.1677	419

*One unit of activity is defined as $1x10^8$ effective molecules.
+C_1q determined spectrophotometrically using $E^{1\%}_{280}$ of 0.682.

was conducted according to Weber and Osborn.[21] For electrophoresis under non-reducing conditions, iodoacetamide (0.05 M) was added while reduced conditions were achieved by the addition of dithiothreclol (10 mM) without iodoacetamide. After electrophoresis, the gels were fixed in 10% TCA for half an hour and stained with 0.25% Coomassie brilliant blue in methanol, acetic acid and water (10:1:9 v/v) for 20 hours and destained by 7.5% acetic acid containing 5% methanol.

RESULTS

Table 1 shows C_1q recovery and the purification factor at different steps. Euglobulin precipitation by 5 mM ethylenediamine, pH 8.8 after titrating the sera containing 5 mM EDTA to pH 8.7 to 8.9, resulted in a 140-fold increase in specific activity with 74 per cent recovery of C_1q. Under these conditions, immunoglobulin contaminates (Table 2) which were away from their isoelectric point (IpH) at this pH remained soluble, whereas C_1q which was close to its IpH was precipitated from the sera.

Chromatography of euglobulin material on the $RAHC_1q$ column (Fig. 1) and RAHNS column (Fig. 2) resulted in a highly enriched preparation of C_1q with a 492- and 427-fold purification of protein along with a total recovery of 69 per cent and 41 per cent respectively from the two columns (Table 1). The recovery of C_1q from the RAHNS column was very poor which may have been due to the presence of trace amounts of anti-C_1q which we were unable to detect otherwise in our anti-NHS.

Table 2 Characterisation of other immunoglobulins during purification.

Procedure	IgG	IgA	IgH	C_1q*
NHS	+++	+	++	+
RAH C_1q column flow-through	+	–	+	±
RAH C_1q column eluant	–	–	–	+++
RAH NS column flow-through	+	–	±	+++
RAH NS column eluant	++	–	+	±

*C_1q assayed by rocket immunoelectrophoresis.

PAGE analysis of the pooled fractions containing C_1q gave a single band (Fig. 3b) in the case of the $RAHC_1q$ column while two bands (Fig. 3C) were seen when eluted from the RAHNS column due to the contaminating IgG (Table 2), elimination of the IgG from the C_1q preparation was efficiently accomplished on the GAH IgG column at 1.0 M NaCl (Fig. 4). PAGE of the eluted protein gave only one band corresponding to IgG (Fig. 3d). In high salt concentrations, C_1q is unable to bind to solid-phase GAHIgG, whereas the antibody activity for human IgG is not affected. Thus, C_1q passes in the flow-throughs whereas contaminating human IgG is retained.

Similarly, we have used 1 M NaCl for SRID or rocket IEP, since C_1q precipitates with preformed immune complexes or heat-aggregated IgG[7] and antibodies dissociated in semi-solid agar at 56°C, may cause heat aggregation giving false positive results due to C_1q precipitation.

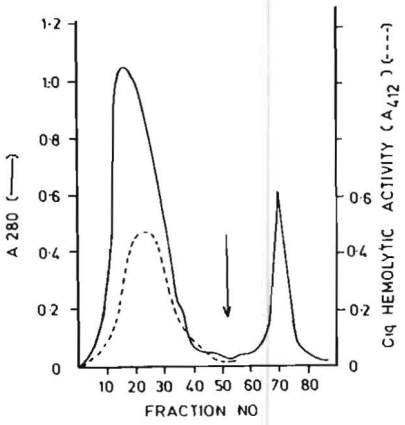

Fig. 1 Rabbit anti-human C_1q Sepharose-4B affinity column chromatography. Flow rate was 20 ml/h & 1 ml fractions were collected. The arrow indicates elution with 1 M NaCl in 0.01 M phosphate buffer containing 0.05 M EDTA, pH 7.5. Fractions were assayed for protein and C_1q haemolytic activity.

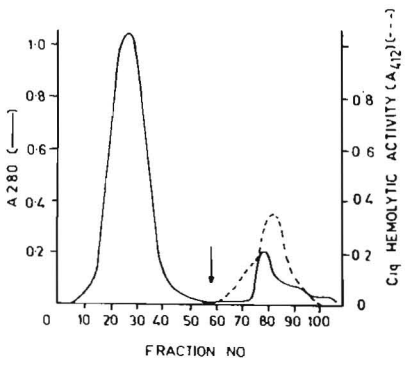

Fig. 2 Rabbit anti-normal human serum (C_1q depleted) Sepharose-4B column chromatography. The column was pre-equilibrated with 1 M NaCl in 0.01 M phosphate buffer containing EDTA (0.01 M). Bound protein was eluted with (arrow) 40% ethylene glycol. The flow-through material contained a small amount of IgG.

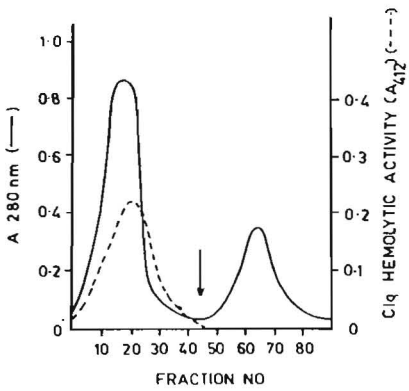

Fig. 4 Rabbit anti-human IgG Sepharose-4B chromatography of flow-through of the RAHNS column. The arrow indicates the start of 40% ethylene glycol. The eluted peak was demonstrated to be IgG.

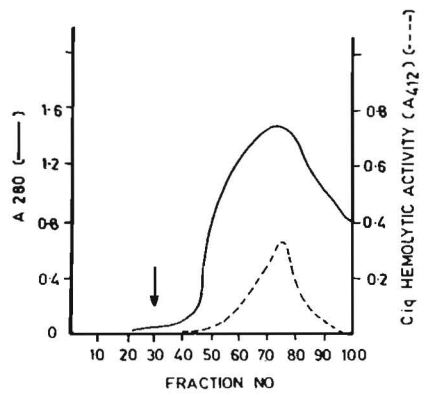

Fig. 6 Con A Sepharose-4B column was equilibrated with Con-A buffer; flow rate was 5 ml/hr with 1 ml fractions collected. Increase in absorbant at 280 nm is due to presence of αMGP in the elution buffer.

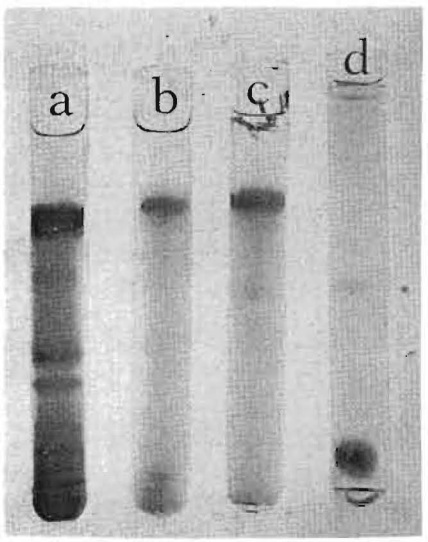

Fig. 3 Polyacrylamide gel electrophoresis of (a) euglobulin precipitate (b) rabbit anti-human C_1q Sepharose-4B eluate, (c) rabbit anti-human normal serum flow-through and (d) eluted peak of rabbit anti-human IgG Sepharose-4B column.

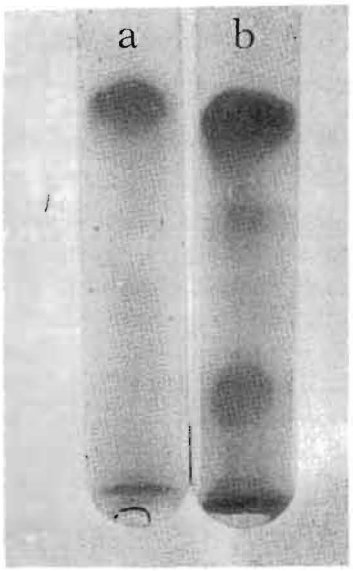

Fig. 5 SDS-Polyacrylamide gel electrophoresis. Electrophoresis of purified C_1q was performed on 9% acrylamide gel under (a) non-reducing and (b) reducing (DTT) conditions.

Ouchterlony analysis of the C_1q preparation revealed a heavy precipitation line with anti-C_1q, but no precipitation line was evident when tested against anti-IgM, IgA, IgG or NHS. IEP analysis showed one line with anti-NHS which was immunologically identical to that produced by anti-human C_1q. No IgG or IgM was detected. SDS-PAGE analysis (Fig. 5) under non-reducing conditions produced two bands (Fig. 5a) while three bands were observed (Fig. 5b) upon reduction with DTT. The C_1q preparation at this step was practically homogeneous although no functional test was performed to determine the presence of other complement components. These results suggest no contamination since no other band was detected on PAGE even at a high level of loading. The yield of C_1q was approximately 14-20 mg from 1 litre of serum.

This preparation was then applied to a Con A Sepharose column (Fig. 6) as described by Kolb, Kolb and Padack,[8] but no separation was achieved and we could not detect any inhibitor in the flow-through fractions.

DISCUSSION

Since C_1q has minimal solubility in low ionic strength buffers and since EDTA dissociates the C1 macromolecule into its constituents, this property has been utilised by many workers to prepare euglobulin precipitate[4,5] but this results in considerable immunoglobulin contamination. The preparation of euglobulin at an alkaline pH after titrating 5 mM EDTA serum to pH 8.7-8.9 resulted in complete C_1q precipitation, whereas immunoglobulin contamination, which is away from IpHs under these conditions, remains soluble. Also, for euglobulin prepared at this pH, lipid contamination is almost nil.

Pohl and coworkers[11] have reported a 505-fold purification with 52 per cent yield of C_1q from human plasma, whereas Kolb *et al*[8] have reported a 833-fold purification of protein with only a 40 per cent yield by a method involving Sepharose human IgG and Biogel A-5 M. In the present report, yields of 41-69 per cent of the C_1q present in serum were obtained with a 420-490-fold purification factor. These values are similar to those obtained by Angello and coworkers[7] and somewhat lower than those reported by Tenner and coworkers.[12]

In addition to high yields of active C_1q, the present method is comparatively simple and rapid. Affinity chromatography on Sepharose RAHC_1q or Sepharose RAHNS (C_1qD) followed by Sepharose GAH IgG column yielded homogeneous C_1q. However, the yields of C_1q were much better on Sepharose RAH C_1q so it may be concluded that this matrix can be efficiently used for larger yields of pure C_1q. Neither IgM nor IgG, frequent contaminants of C_1q preparations, was detectable in the final preparation by immunochemical analysis or by PAGE. However, considerable loss of C_1q occurred during the concentration of various fractions, in part due to the formation of irreversible insoluble aggregates.

Conradie *et al*[22] have described a proteoglycan inhibitor in human serum. Silvestri *et al*[23] have characterised it as a naturally occurring chondroitin sulphate proteoglycan inhibitor (MW 800,000 to several million) which can form complexes with C_1q in human serum and which can co-precipitate with C_1q during purification procedures. Kolb *et al*[8] have separated this inhibitor from C_1q by the Con A affinity column in the presence of 0.65 M NaCl. We failed to achieve this separation even in the presence of 1 M NaCl. This can be explained on the basis that C_1q, also being a proteoglycan, will bind to a Con A bed along with an inhibitor and that it co-elutes along with C_1q inhibitor by 10 per cent MGP.

REFERENCES

1. Porter RR, Reid KBM. The Biochemistry of complement. Nature 1978; 275:699-703.
2. Wehler C, Andrews JM, Bing DH. The use of solid phase C_1q and horseradish peroxidase conjugated goat anti-IgG for the detection of immune complexes in human serum. Mol Immunol 1981; 18:157-62.
3. Stimson WH, McAclam A, Hutchison RS. An assay for antigen antibody complexes in human sera using C_1q-enzyme conjugates. J Clin Lab Immunol 1981; 5:129-31.
4. Yonemasu K, Strond RM. C_1q: Rapid purification of monospecific antisera and for biochemical studies. J Immunol 1971; 106:304-13.
5. Volankis JE, Strond RM. Rabbit C_1q: Purification, functional and structural studies. J Immunol Methods 1972; 2:25-34.
6. Sledge CR, Bing DH. Purification of the human complement protein C_1q by affinity chromatography. J Immunol 1973; 111:661-6.
7. Angello U, Winchester RJ, Kunkel HG. Precipitin reactions of the C_1q component of complement with aggregated γ-globulin and immune complex in gel diffusion. Immunology 1970; 19:909-19.
8. Kolb WP, Kolb LM, Padack ER. C_1q: Isolation from human serum in high yield by affinity chromatography and development of a highly sensitive hemolytic assay. J Immunol 1979; 122:2103-11.
9. Lin TT, Fletcher DS. Interaction of human C_1q with insoluble immunoglobulin aggregates. Immunochemistry 1978; 15:107-17.
10. Paul SM, Liberti PA. Isolation and characterization of highly stable rabbit C_1q. J Immunol Methods 1978; 21:341-53.
11. Pohl DA, Gibbons JJ Jr, Tsai CC, Roodman ST. Isolation and purification of human C_1q from plasma. J Immunol Methods 1980; 36:13-27.
12. Tenner AJ, Lesavre PH, Cooper NR. Purification and radiolabelling of human C_1q. J Immunol 1981; 127:648-53.
13. Williams CA. In: Williams CA, Chase MW, eds, Methods in immunology and immunochemistry. New York: Academic Press, 1971; 3:234-41.
14. Ouchterlony O. Diffusion-in-gel methods for immunological analysis. In: Kallos P, Waksman BH, eds, Progress in Allergy, Vol VI. New York: Karger 1981:30-154.
15. Mancini G, Carborana AO, Hermans JC. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 1965; 2:235-54.
16. Laurell CB. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Ann Biochem 1966; 15:45-52.
17. Reid KBM, Lowe DM, Porter RR. Isolation and characterization of C_1q, a subcomponent of the first component of complement from human and rabbit sera. Biochem J 1972; 130:749-63.
18. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265-75.
19. Borsos T, Rapp HJ, Mayer MM. Studies on the second component of complement. J Immunol 1961; 87:310-25.
20. Davis BJ. Disc electrophoresis-II. Method and application to human serum proteins. Ann NY Acad Sci 1964; 121:404-26.
21. Weber K, Osborn MJ. The reliability of molecular weight determinations by dodecyl sulphate – polyacrylamide gel electrophoresis. J Biol Chem 1969; 244: 4406-12.
22. Canradie SD, Volanakis JE, Stroud RM. Evidence for a serum inhibitor of C1q. Immunochemistry 1975; 12:967-71.
23. Silvestri LJ, Baker J, Roden L, Stroud RM. Paper presented at the Arthritis foundation, American Rheumatism Association section meeting, New Orleans, La, Dec. 1, 1978 (Abstract No. 2).

Immunochemical Characterisation of Cryoglobulins*

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SUMMARY **An immunochemical characterisation of four cryoproteins is presented. Two of them were typical "mixed" cryoglobulins with two protein peaks on gel filtration at low pH values, the first peak was monoclonal IgM kappa in one and monoclonal kappa IgA in the other. The other two cryoproteins were unusal. One of them showed only one protein peak on gel filtration; it was monoclonal IgG kappa. The other cryoprotein showed the first peak of aggregated gammaglobulins and the second peak of gamma heavy chains devoid of light chain activity. Functional analysis revealed rheumatoid factor activity in three of the cryoproteins and antinuclear antibody in one of them. It is concluded that all four of the cryoproteins were "mixed" in nature but that two of them were unusual in nature.**

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While cryoglobulinaemia is occasionally seen as a primary or idiopathic disease, it is most often associated with monoclonal gammopathies, Raynaud's phenomenon and purpura.[1] Recently, it has become apparent that a small amount of cryoglobulin can also occur in other diseases, most often associated with collagen diseases, glomerulonephritides and generalised vasculitides.[2-5]

Cryoglobulins appear to fall within the two general categories. In one group, usually two immunoglobulin classes are present, one acting as the antibody directed against the other which acts as the antigen.[6] The other type of cryoglobulin includes those which usually have no antibody activity but exhibit increased inter-molecular attraction at low temperatures. A single homogenous immunoglobulin component is found in this category.[7,8]

In this report, we present the data on the analysis of four cryoproteins.

MATERIALS AND METHODS

In a routine screening for the cryoproteins in patients with clinical features of cryoproteinaemia, four cryoproteins were obtained in significant amounts enabling detailed analysis. For convenience they were labelled cryo-1, cryo-2, cryo-3 and cryo-4.

Isolation of cryoprotein

The cryoproteins were isolated from the serum obtained by allowing blood to clot at 37°C for 1-2 hours. Serum was kept at 4°C and observed for up to 10 days for the appearance of chalky precipitate or gellification. If cryoproteins were observed, the serum was centrifuged at 4,000 rpm at 4°C for 30 minutes and the cryoprecipitate was washed twice with cold 0.1 M phosphate buffered saline (PBS) at 4°C. In each case, the cryoprecipitate could not be solubilised in neutral or slightly alkaline buffer (pH 7.0 to 8.0 at room temperature). However, it could be solubilised in 0.1 M sodium acetate buffer, pH 4.0 at room temperature. The experiments with a native cryoprotein solution were, therefore, conducted under these conditions unless otherwise stated.

Separation of cryoprotein components

The reconstituted cryoglobulin was fractionated on Biogel A-1.5 M column (Biorad, Richmond, Cal., U.S.A.) (15x2.5 cm) prequilibrated with 0.1 M sodium acetate buffer, pH 4.0. Fractions were monitored spectrophotometrically at 280 nm, elution peaks were pooled, concentrated, dialysed against PBS and analysed immunochemically for

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precipitation with isolated IgG and for rheumatoid factor and anti-nuclear antibody activity.

Immunological characterisation

Immunoelectrophoresis was carried out using 1.5% Noble agar (Difco, Detroit, Mich, U.S.A.) in 0.05 M veronal buffer (pH 8.6) and a field strength of approximately 2 mA per slide. Light chain activity was screened by Ouchterlony's double diffusion in 1.5% agarose in veronal buffer as above. Heavy chain specific rabbit anti-human IgG, IgA and IgM and human anti-kappa and anti-lambda light chain antisera were obtained from Cappel Lab, West Chester, Pens, U.S.A. Anti-whole human serum was prepared in our laboratory.

Anti-nuclear antibody (ANAB) was screened in the whole serum and isolated cryo-components by standard immunofluorescence technique.[9] Rheumatoid factor was detected by passive agglutination of latex particles sensitised with human IgG.[10] The test was carried out both at 37°C and 4°C with whole serum and isolated cryo-components.

The effect of cross-mixing of cryo-components with normal immunolobulin was studied by mixing equal amounts (20 μl each) of the isolated fractions with IgG purified on the DEAE cellulose column (2x 20cm) pre-equilibrated in PBS and eluted by increasing the salt concentration (0.005 x 0.2 M). The resulting precipitate was assessed visually and tested for its cryo-precipitability by warming to 37°C. The protein content of cryoprotein was estimated quantitatively by using Folin phenol reagent.[11] Immune complexes were detected by the C_1q radiolabelled binding assay test.[12] Polyacrylamide gel electrophoresis (PAGE) was conducted using 7.5% gel in 0.1 M tris-glycine buffer.[13] The gels were stained with 0.25% Coomasie Brilliant Blue in methanol: acetic acid: water [5:1:5 (v/v)] and destained with 7% acetic acid.

RESULTS

The gel filtration analysis(Fig. 1) of the four cryoproteins revealed two protein peaks in cryo-1, cryo-2 and cryo-4 but only one peak in cryo-3. Immunochemically, the first protein peak in cryo-1 and cryo-2 was the kappa type monoclonal IgM and IgA, respectively; the second peak was polyclonal IgG in both cases (Fig. 3). The elution profile of cryo-4 was rather peculiar (Fig. 2). A small peak in void volume was probably the undissociated cryoprotein complex which preceded the second peak. The second peak was devoid of any light chains but showed a gamma heavy chain and a trace of alpha heavy chain which were demonstrable by double diffusion in agar with specific antisera (Fig. 3). The first peak showed the presence of immune complexes and was equivalent to 4.97 mg of aggregated human gamma globulin as detected by C_1q binding assay. Figure 4 shows a characteristic cryoglobulin electrophoresed on PAGE. The cryoprecipitate (Fig. 4b) contains approximately five to six as yet unidentified bands including a few other components of normal serum basides immunoglobulins. The analysis of rheumatoid factor activity in the whole serum and in different isolated peaks of cryoproteins is given in Table 1. The whole

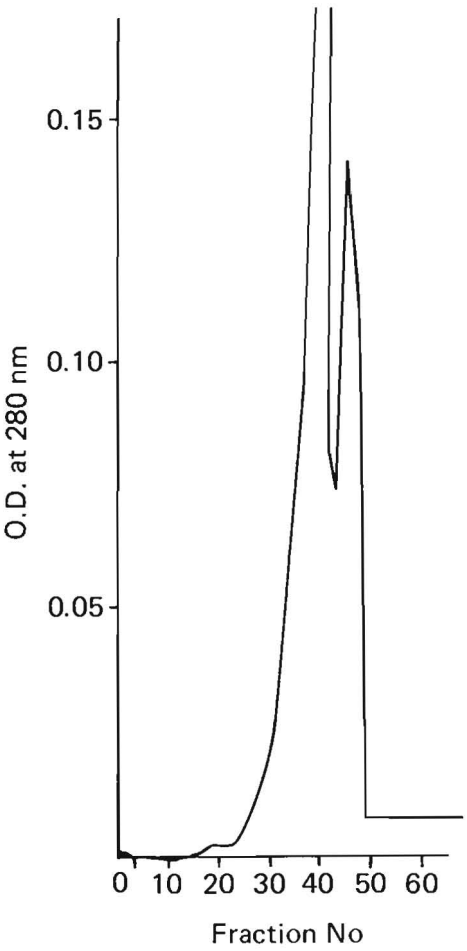

Fig. 1 Elution pattern (Biogel A-1.5 M) of a reprasentative isolated cryoglobulin. Elution buffer was sodium acetate (0.1 M), pH 4.1 with a fraction volume of 3 ml.

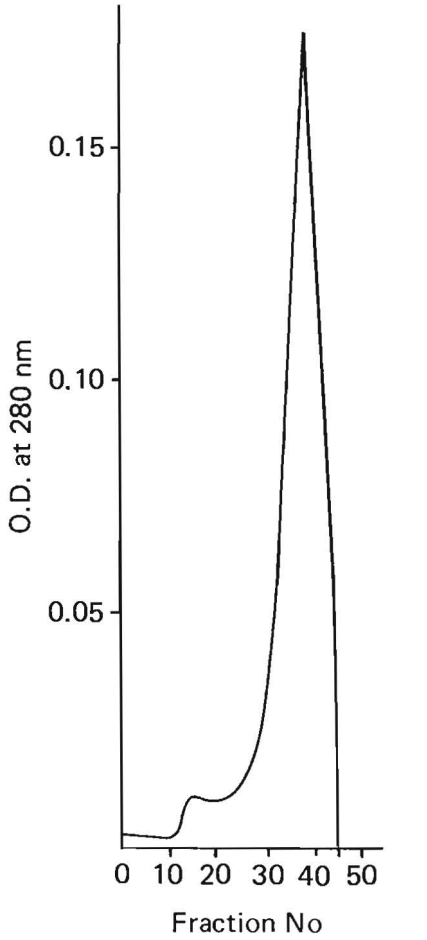

Fig. 2 Elution profile of cryoprecipitate of cryo-4. (Note small peak in void volume).

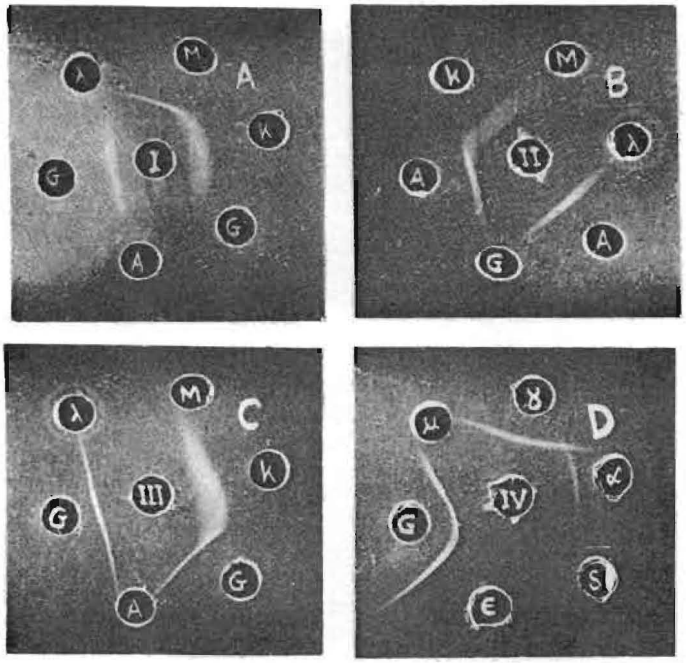

Fig. 3 Oucterlony double diffusion analysis for isolated first peak – centre well (A) cryo-1, (B) cryo-2, (C) cryo-3 and (D) cryo-4. Side well-G-anti IgG; M-anti-IgM; A-anti-IgA; kappa-anti-knppa light chain and lambda-anti-lambda light chain. Gamma, mu, alpha, delta and epsilon are heavy chain specific antisera to anti-IgG, anti-IgM, anti-IgA, anti-IgD and anti-IgE, respectively (cross reactivity in between anti-IgE and anti-whole-IgG was due to species specificity).

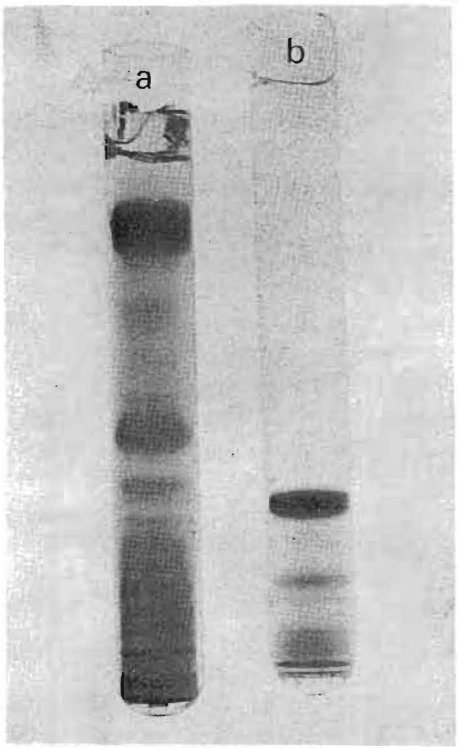

Fig. 4 Polyacrylamide gel electrophoretic pattern of (a) whole cryoglobulinaemic serum (case III) and (b) isolated cryoglobulin of the same serum.

Table 1 Immunochemical characterisation of isolated peaks

	Cryoprotein concentration (mg/ml)	Whole serum		1st peak					2nd peak		Characterisation	Classification
		RF	ANAB	Immunoglobulin	Light chain	ANAB	RF 37°C	RF 40°C	Immunoglobulin	Light chain		
Cryo-1	1.1	+	–	IgM	k	–	–	++	IgG	k	Monoclonal IgM, kappa type with polyclonal IgG	Type II
Cryo-2	0.55	+	–	IgA	k	–	–	+	IgG	k	Monoclonal IgA, kappa type with polyclonal IgG	Type II
Cryo-3	4.5	+	–	IgG	k	–	–	++	–	–	Monoclonal IgG, kappa type	Type II
Cryo-4	0.6	–	+	–	–	+	–	–	IgG	missing	Gamma heavy chain with trace of alpha heavy chain	Type II

serum of cryo-1, cryo-2 and cryo-3 showed rheumatoid factor activity while cryo-4 serum was negative. Rheumatoid factor activity was also demonstrable in the first peak of cryo-1 and 2, but only at 4°C. The single peak of cryo-3 also showed RF activity. No RF activity was demonstrable in any of the second peaks of any of the cryoproteins, neither at 4°C nor at 37°C. As the presence of RF activity has been shown to be a definite indicator of "mixed" nature of cryoproteins,[14] cryo-1, 2 and 3 could be labelled as "mixed" cryoglobulins.

The first three cryoglobulinaemic sera and isolated cryoproteins gave negative results in the indirect immunofluorescence test for antinuclear antibody. However, cryo-4 showed positive ANF which was also present in the first peak of fractioned cryoproteins. Considering that auto-antibody activity is usually seen in mixed cryoproteins, even cryo-4 would be considered as a "mixed" cryoprotein in spite of having a monoclonal component without rheumatoid factor activity.

Since three of the cryoglobulins consisted of a mixture of IgM, IgA or IgG globulin with polyclonal IgG and possessed RF activity in the para-protein component, attempts were made to determine whether IgG, IgM and IgA or both fractions were necessary for cryoprecipitability and RF activity. The isolated components did not exhibit cold insolubility separately. Recombination (20 μl each) again produced a cryoprecipitate. Recombination of monoclonal component with isolated IgG from human serum also produced a precipitate which was insoluble on heating to 37°C. The reason for this could be that isolated IgG may be partially denatured whereas the native state may be essential for the reversibility of the phenomenon.

DISCUSSION

Immunochemically, cryoproteins are classified into three categories.[14-17] Type I is monoclonal cryoglobulin made of immunoglobulins with only one class or subclass of heavy and/or light chains. Type II is mixed cryoglobulinaemia with components made of immunoglobulin belonging to two different classes, one of which is monoclonal. Type III is polyclonal mixed cryoglobulin which comprises a heterogeneous immunoglobulin molecule belonging usually to two or more different classes and sometimes is made of additional serum proteins.

In the present study, cryo-1 and 2 were the straightforward type II cryoglobulins, as they showed a monoclonal component (IgM in case I and IgA in case II) with polyclonal IgG. Previous reports also mention that in cryoglobulinaemia, the first component is usually either monoclonal IgM,[18] IgA[19] and IgG[20] or a polyclonal immunoglobulin.[2,21]

Cryo-3 apparently had only a monoclonal component. Thus, on gel filtration it appeared that this cryoprotein was a type I cryoprotein which is usually seen in cases of multiple myeloma. However, the clinical features and follow-up observations of the patient with cryo-3 were not consistent with the diagnosis of multiple myeloma. On the other hand, functional studies were compatible with essential mixed cryoglobulinaemia because the monoclonal component was showing antiglobulin (RF) activity (Table 1). It is possible that, in such cases, part of the IgG population behaves like an antigen while the other IgG molecules represent antibody.[22] Thus, cryo-3 may belong to a very rare category called essential mixed monoclonal cryoglobulin. There is a report of a patient who was initially described as having essential cryoglobulinaemia but after 15 years he developed multiple myeloma from a benign condition.[23]

Cryo-4 was unusual because, in spite of the low pH, complete separation of the different components of the cryoprotein was not effected. The reason for this is not clear. The second peak was devoid of light chains and showed the presence of more than one heavy chains thus indicating the mixed nature of the cryoprotein. However, it has been pointed out by Skravil and Barandun[24] that for the detection of light chain activity in the agar double diffusion test, a very precise balance of antigen and anti-serum is necessary. Therefore, the possibility of a technical difficulty in demonstrating the light chain in cryo-4 cannot be excluded. Whole serum of cryo-4 was positive for the DS-DNA antibody which could not be traced out in isolated components because of low concentration. Therefore, cryoprecipitation of cryo-4 might have been dependent either on non-covalent bonds or on some unique antigen present in the cryoglobulin itself.

Results of cross-mixing studies involving cryoproteins and normal immunoglobulin are suggestive of immunological interaction. Moreover, since the paraprotein alone is not cryoprecipitable and since it co-precipitates with IgG, it appears that the paraproteins act as autoantibodies to IgG thus representing an antigen-antibody complex formed at low temperature.[25] This is in concurrence with the suggestion of Wager and coworkers that autologous IgG may become auto-antigenic after reacting with a primary antigen or antigen-antibody complex.[26] The immune system of the host would then respond to these pathogenic complexes (primary antigen/IgG antibody) by producing a corresponding antibody. It is also possible that their prescence is coincidental and of no immunopathogenic significance.

The ambient temperature in our country generally is not very low; therefore, clinical symptoms of cryoproteinaemia usually do not appear. A careful screening for cryoproteinaemia in the suspected clinical conditions may be helpful

both for diagnosis and may also give a clue to the possible mode of treatment including plasmapheresis. Primary over-production of cryoglobulin may occur in cases of myeloma and other lymphoproliferative diseases whereas secondary cryoglobulinaemia develops with infections, auto-immune diseases and collagen vascular diseases. Besides these practical implications, the study of cryoglobulin is extremely interesting as it yields excellent information regarding the reactivities of different immunoglobulins.

REFERENCES

1. Churchill WJ Jr, Ham NL. Type III cryoglobulinemia with necrotizing vasculitis involving skin, intestine and glomeruli in malignant lymphoma of non-Hodgkin type. N Eng J Med 1981; 304:774-7.
2. Grey HM, Kohler PF. Cryoglobulins. Seminar Hematol 1973; 10:87-112.
3. McPhaul JJ Jr. Cryologulinaemia in patients with primary renal disease and systemic lupus erythematosus-IgG and DNA-binding assessed by cryoprecipitation. Clin Exp Immunol 1978; 31:131-40.
4. Cryer PE, Kissane JM. Cryoglobulinaemia. Am J Med 1980; 68:757-64.
5. Popp JW Jr, Diestang JC, Block JK. Essential mixed cryoglobulinemia without evidence for hepatitis virus infection. Ann Intern Med 1980; 92:379-483.
6. Meltzer M, Franklin EC. Cryoglobulinemia – A study of twenty-nine patients. I. IgG and IgM cryoglobulins and factors affecting cryoprecipitability. Am J Med 1966; 40:828-36.
7. Saha A, Edwards MA, Sargent AU, Rose B. Mechanism of cryo-precipitation I. Characteristics of human cryoglobulin. Immunochemistry 1968; 5:341-50.
8. Anderson BR, Tesar JT, Schmid FR, Haisty WK, Hartz WH Jr. Biological and physical properties of a human M-cryoglobulin and its monomer subunit. Clin Exp Immunol 1971; 9:795-807.
9. Holborow ET, Johnson GD. Immunofluorescence: In: Weir DM, ed, Hand book of experimental immunology. Oxford: Blackwell Sci Pub, 1967:571-9.
10. Singer JM, Plotz CM. The latex fixation test. Am J Med 1956; 21:886-92.
11. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193:265-75.
12. Zubler RH, Lange G, Lambert PH, Hiescher PA. Detection of immune complexes in unheated sera by modified ^{125}I-C_1q binding test: Effect of heating on the binding of C_1q by immune complexes and application of the test to systemic lupus erythematosus. J Immunol 1976; 166:232-5.
13. Davis BJ. Disc electrophoresis-II. Method and application to human serum proteins. Ann NY Acad Sci 1984; 121:404-26.
14. Brouet JC, Clauvel JP, Danon F, Klein M, Seligmann M. Biologic and chemical significance of cryoglobulins-report of 86 cases. Ann J Med 1974; 57:775-88.
15. Barnett EV, Cryoglobulinemia and disease. Ann Intern Med 1970; 73:95-107.
16. Gorevic PD, Kassab HJ, Levo Y, et al. Mixed cryoglobulinemia: clinical aspects and long-term follow-up of 40 patients. Am J Med 1980; 69:287-308.
17. Levo Y. Nature of cryoglobulinaemia. Lancet 1980; 1:285-6.
18. Sione MJ, Fedak JE. Studies on monoclonal antibodies. J Immunol 1974; 113: 1377-85.
19. Whitsed HM, Penny R. IgA/IgG cryoglobulinemia with vasculitis. Clin Exp Immunol 1971; 9:183-91.
20. Grey HM, Kohler PF, Terry WD, Franklin DC. Human monoclonal G-cryoglobulins with anti-globulin activity. J Clin Invest 1968; 47:1875-84.
21. Cordonnier D, Martin H, Groslamber LP, Micovin C, Chenais F, Stosbner P. Mixed IgG-IgM cryoglobulinemia with glomerulonephritis. Am J Med 1975; 59:867-71.
22. Cerottini JG, Grey HM. Binding properties of monoclonal G – Antiglobulin factors with human IgG. Ann NY Acad Sci 1969; 168:76-92.
23. Gordon-Smith EC, Harrison J, Hobbs JR. Multiple myeloma presenting as cryoglobulinemia. Proc Roy Soc Med 1968; 61: 1112-5.
24. Skvaril F, Barandun S. A simple method for simultaneous detection of human immunolobulins of both light chain types. J Immunol Method 1973; 3:127-36.
25. Meltzer M, Franklin EC, Elias K, McCluskey RT, Cooper N. Cryoglobulinemia – a clinical and laboratory study. II. cryoglobulins with rheumatoid factor activity. Amer J Med 1966; 40:837-56.
26. Wager O, Rasanen JA, Hagman A, Klemola E. Mixed cryoglobulinemia in infections mononucleosis and cytomegalovirus mononucleosis. Int Arch Allerg Appl Immunol 1968; 34:345-61.