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ORIGINAL ARTICLES

Quantitation of Isotype Specific *Haemophilus influenzae* Antibody in Serum and Saliva of Normal Subjects and Chronic Bronchitics

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SUMMARY An immunoglobulin isotype specific radioimmunoassay procedure has been developed to assess the antibody response to *Haemophilus influenzae* somatic antigens in serum and mucosal secretions. This assay was reproducible (between assay CV% 13.9; within assay CV% 4.5 IgG, 3.9 IgA, 3.0 IgM) and specific for H_1/H_2 antigens. Different patterns of antibody were observed in healthy children (aged 5-10 years), adults and patients with chronic bronchitis. In serum, 20% of chronic bronchitics had antibody levels greater than those observed in healthy adults. In saliva, the proportion of chronic bronchitic patients with high levels (greater than 12% binding) of IgG specific antibody was significantly greater ($P < 0.05$) than in healthy adults or children. The proportion of children and chronic bronchitics which had antibody levels of up to 4% binding was significantly greater ($P < 0.05$) than that observed in healthy adults. A similar pattern was observed for IgM specific antibody. The occurrence of IgA specific antibody in the saliva in children and chronic bronchitics was consistently greater than that observed in adults for all levels of antibody ($P < 0.05$). Chronic bronchitics with high levels of antibody had greater infection and mortality rates.

The role played by *Haemophilus influenzae* in the pathogenesis of chronic bronchitis and its complications remains controversial 30 years after May first associated this bacteria with acute purulent exacerbations of bronchitis.[1] This, in part, reflects a failure to adequately assess the host response to *H. influenzae* in normal subjects as well as chronic bronchitics, due to lack of a sensitive, specific and quantitative assay capable of detecting antibody in both serum and mucosal secretions. Many previous studies have used crude antigen preparations which cross react with other gram negative bacteria[2] and techniques which were insensitive, semiquantitative, and incapable of quantitating antibody isotype.[2-7] Although some attempts have been made to define the antibody isotype specific for *H. influenzae* by immunofluorescence and radioimmunoelectrophoresis,[3,8-10] these studies do not satisfactorily define the humoral immune response to *H. influenzae* in chronic bronchitis. Davies *et al* separated *H. influenzae* cytoplasmic antigens into specific (H_1 and H_2) and non specific (H_3, H_4, and H_5) components,[4] which have been used to demonstrate precipitins in sera from 50-60% of patients with chronic bronchitis compared with less than 16% of controls.[7,9,10] However, the inability of this assay procedure to quantitate the antibody response limits its value in interpreting differences between chronic bronchitics and normal subjects. We have therefore developed a sensitive, class specific radioimmunoassay which detects antibody to H_1/H_2 antigen of *H. influenzae* in serum and mucosal secretions.

MATERIALS AND METHODS

Subjects

The sera and saliva of 40 healthy adults (18-40; mean 31 years), 76 chronic bronchitics and the saliva of healthy children (20 five-year-olds; 25 ten-year-olds) were assayed for the presence of specific anti-*H. influenzae* antibody. The patient group all had obstructive chronic bronchitis as defined by the M.R.C. of Great Britain.[11] The age range was 44-85 (mean 64 years), and the average duration of symptoms was about 10 years. Lung function was assessed by standard spirometry.

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Episodes of infection were assessed according to an increase in volume and purulence of sputum, with fever.

Specimen collection

Saliva samples were obtained according to the method of Ostergaard and Blom.[12] Samples were collected 2 hours post prandially when there is least variation in salivary flow rates and proteins (Unpublished observations). Following collection, the saliva was frozen at-20°C for 24 hours, thawed and then cellular debris and mucous was removed by centrifugation at 600 g for 10 min. Blood samples were collected by venepuncture. Saliva from which debris and mucous were removed and serum specimens were stored at -70°C until assayed. All samples were taken at times when no clinical infection was present.

Culture conditions and antigen preparation

A non-capsulated strain of *H. influenzae* was obtained from the Swiss Serum and Vaccine Institute, Berne, Switzerland. Brain heart infusion broth containing 300 μg/ml bacitracin was inoculated under sterile conditions and cultured at 37°C in air containing 5% O_2 for 48 hours. Subcultures were then made onto chocolate agar plates containing 300 μg/ml bacitracin and cultured for 24 hours. The bacteria were then harvested by washing into sterile 0.15 M NaCl and washed twice in the same. The organisms were finally resuspended in Michaelis buffer (pH 8.0) at a concentration of 10^{10} per ml. H_1/H_2 cytoplasmic antigens were then prepared according to the method of Davies *et al.*[4] Antigenicity was confirmed by gel precipitation using sera from subjects with chronic bronchitis and bronchiectesis.

Radioiodination of H_1/H_2 antigens

H_1/H_2 antigens were radiolabelled with ^{125}I (IMS/30 Radiochemical Centre, Amersham), according to the method of Bolton.[13] The trichloracetic acid precipitable radioactivity was greater than 95%. The specific activity of the radiolabelled antigen preparation was 30 μCi/μg protein.

Characterisation of H_1/H_2 antigens

H_1/H_2 antigen preparations were characterised by 3 procedures: fractionation on a Sepharose 6B (Pharmacia Fine Chemicals, Sweden) column (Fig. 1), SDS-polyacrylamide gel electrophoresis[14] (Fig. 2) and immunoblotting.[15,16]

Radioimmunoassay procedure

A class specific radioimmunoassay was developed. Prior to assay samples of serum and saliva were prediluted 1:10 and 1:2 respectively in 0.15 M phosphate buffer (pH 7.2) containing 0.15 M NaCl and 10 g/l bovine serum albumin (RIA Grade, Sigma, U.S.A.) (PBS). The assay was conducted in low protein absorption tubes (Gelman Sciences, Australia).

To 100 μl of the prediluted test sample 100 μl of radiolabelled antigens were added. The mixture was then vortexed and incubated at 4°C for 18 hours. 100 μl of respective class specific rabbit anti-human Immnobeads (Bio-Rad Laboratories, U.S.A.) were then added as the solid phase, mixed and incubated for 4 hours at 4°C. The beads were then pelleted, washed twice in PBS by centrifugation (800 g for 15 minutes) and bound radiolabel measured in a gamma counter (Rack-Gamma 1275, LBK, Sweden). All assays were performed in triplicate. Background binding measured by substituting 100 μl of test sample with PBS was less than 1.5%. Monospecificity of the respective Immunobeads was confirmed by immunoelectrophoresis and immunofluorescence (Personnel communications: Dr. M. Schroeder, Bio-Rad Laboratories, U.S.A.)

The level of specific anti-*H. influenzae* antibody was calculated by expressing the test result minus the relevant assay blank as a percentage of the total radioactivity added. A known positive and negative control sample was included in each assay run. The coefficient of variation of the between assay runs was 13.9% (n = 12). Results were standardised to the positive control in each assay. The within assay coefficient of variation was 4.5, 3.9 and 3.0% for IgG, IgA and IgM respectively.

Specificity of radioimmunoassay

Inhibition experiments were conducted to determine the specificity of the assay using H_1/H_2 antigens and an antigen preparation of *Escherichia coli*. A crude antigen was prepared from six common enteric. *E. coli* serotypes (075: HNM, 0101: HNM, 0123: H6, 0141: H4, 018ac: H7 and 0149: H10).[17] A known positive sample of serum and saliva was incubated with 100 μl of radiolabelled antigens and 100 μl of cold antigen. Unlabelled H_1/H_2 antigen or *E. coli* antigen was added in over a concentration range of 0 to 5 mg/ml for serum and saliva. The mixture was then incubated for 18 hours at 4°C and the assay continued as described above.

RESULTS

Characterisation of H_1/H_2 antigens

200 ul of radiolabelled antigens were fractionated on a Sepharose 6B column that had been previously calibrated with normal human serum. Figure 1 shows the H_1/H_2 antigens to be of large molecular weight appearing in the exclusion fraction. In Figure 2 the results of SDS-polyacrylamide gel elctrophoresis is shown. Approximately 30 protein bands were identified within the molecular weight range 14,400-

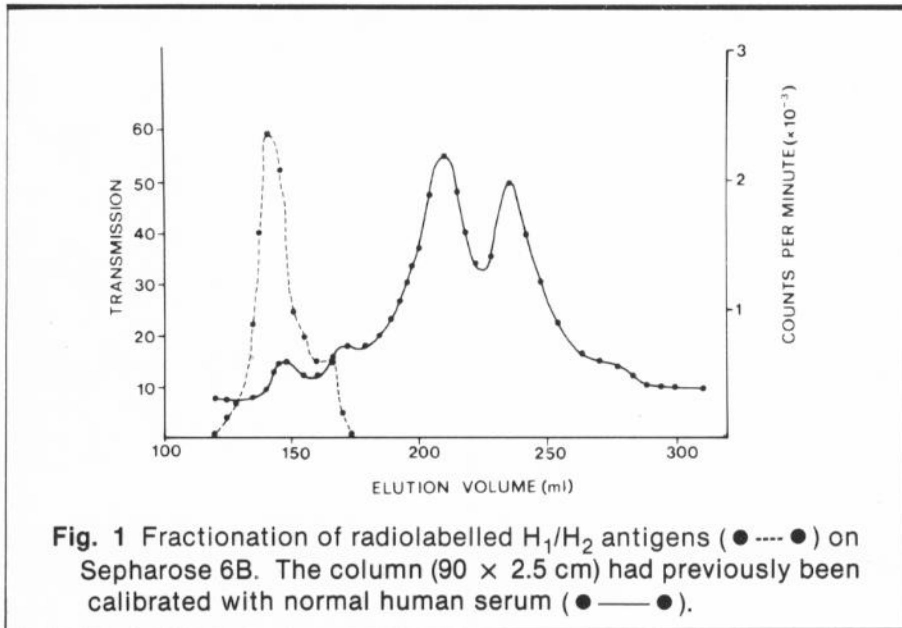

Fig. 1 Fractionation of radiolabelled H_1/H_2 antigens (●----●) on Sepharose 6B. The column (90 × 2.5 cm) had previously been calibrated with normal human serum (●——●).

94,000. After electrophoretic transfer to nitrocellulose paper analysis of the profile using a serum pool prepared from chronic bronchitics revealed IgG antibody reactivity with 14 of the protein bands identified by SDS-polyacrylamide gels. (data not shown). Further analysis of the H_1/H_2 antigens is being conducted.

Specificity of anti-*H.influenzae* radioimmunoassay

Inhibition studies were conducted using an unlabelled antigen preparation for both serum and saliva. As shown in Figure 3, binding could be inhibited by the addition of 1.0 mg/ml of unlabelled H_1/H_2 antigens to saliva and 5.0 mg/ml to serum. No significant inhibition of antibody binding was detected with addition of *E. coli* antigen.

***H. influenzae* antibody in serum**

The frequency distributions of *H. influenzae* antibody in the serum of normal healthy adults and chronic bronchitics is shown in Figure 4. Square root transformation of the data gave a normal distribution to which standard Gaussian statistics were applied. There was no significant difference in the mean level of *H. influenzae* for any

Molecular Weight
94,000
67,000
60,000
43,000
30,000
20,000
14,000

Fig. 2 SDS polyacrylamide gel electrophoresis of H_1/H_2 antigens.

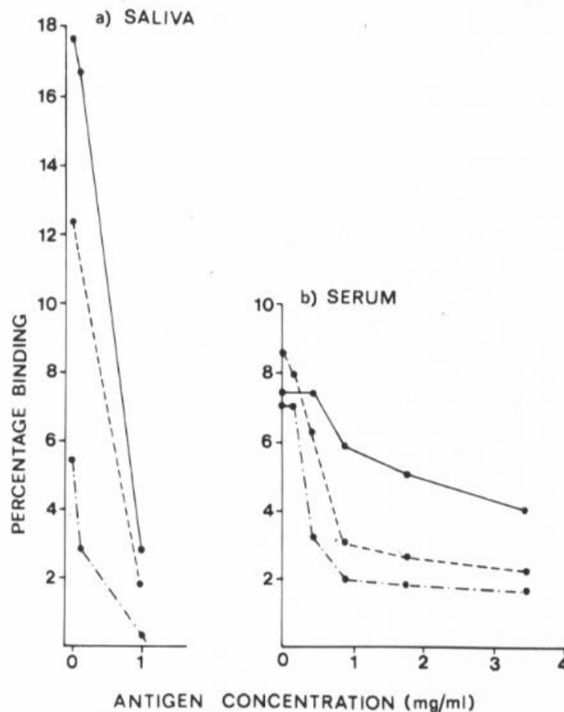

Fig. 3 Inhibition of binding by the addition of unlabelled antigen to the assay system (a) saliva, (b) serum. ●----● IgG; ●——● IgA; ●—·—● IgM.

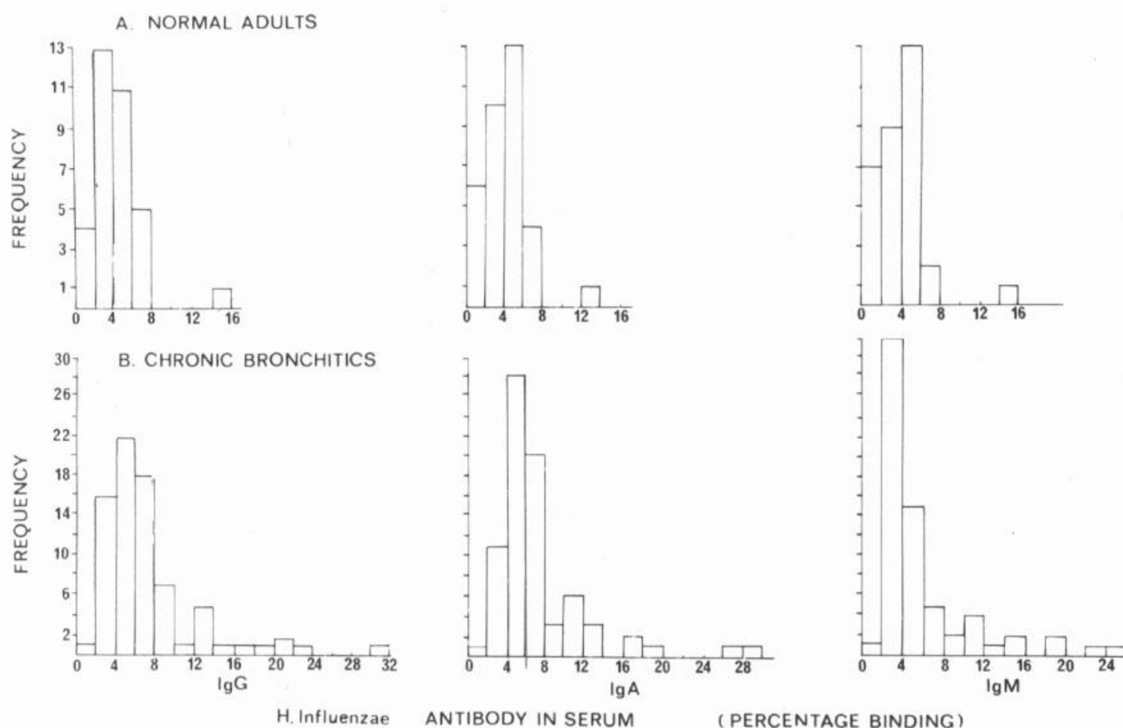

Fig. 4 Frequency distributions of *H. influenzae* antibody in the serum of normal adults and chronic brochitics for the three immunoglobulin isotypes, IgG, IgA and IgM.

immunoglobulin isotype between normal adults and chronic bronchitics. However, 97% of normal adults had levels less than or equal to 8% binding for each isotype, while 27%, 23% and 21% of the chronic bronchitic popula tion had levels of greater than 8% binding for IgG, IgA and IgM antibody respectively (Fig. 4). Thus a small population (about 20% of chronic bronchitic patients) had levels of circulating antibody greater than those found in normal adults.

H. influenzae antibody in the saliva

The frequency distribution of *H. influenzae* antibody in the saliva of normal adults, chronic bronchitics and children aged 5-10 years are shown in Figure 5. The results were not normally distributed. The cumulative frequency for each group was calculated. χ^2 analysis of the number of subjects at various levels of antibody activity were conducted.[18]

The proportion of subjects in the chronic bronchitic group with high levels of IgG specific antibody was significantly greater than in adults or children (10% binding level, $P > 0.05$; 12% binding level, $P < 0.05$). At lower levels (4%) the proportion of children and chronic bronchitics was significantly greater than that observed in the adults ($P < 0.05$). A similar pattern was

Table 1 Correlation of serum IgA antibody with clinical events

	Low serum antibody (≤3.5% binding) 12.5% of subjects tested	High serum antibody (≥8.0% binding) 15% of subjects tested
Deaths over 9 month period	0	4 (36% of deaths in chronic bronchitic group)
Number of infective episodes per subject over-9 month period	1.2	2.0
FFV (l)	0.83 ± 0.18*	0.73 ± 0.14

*Mean ± standard error of mean.

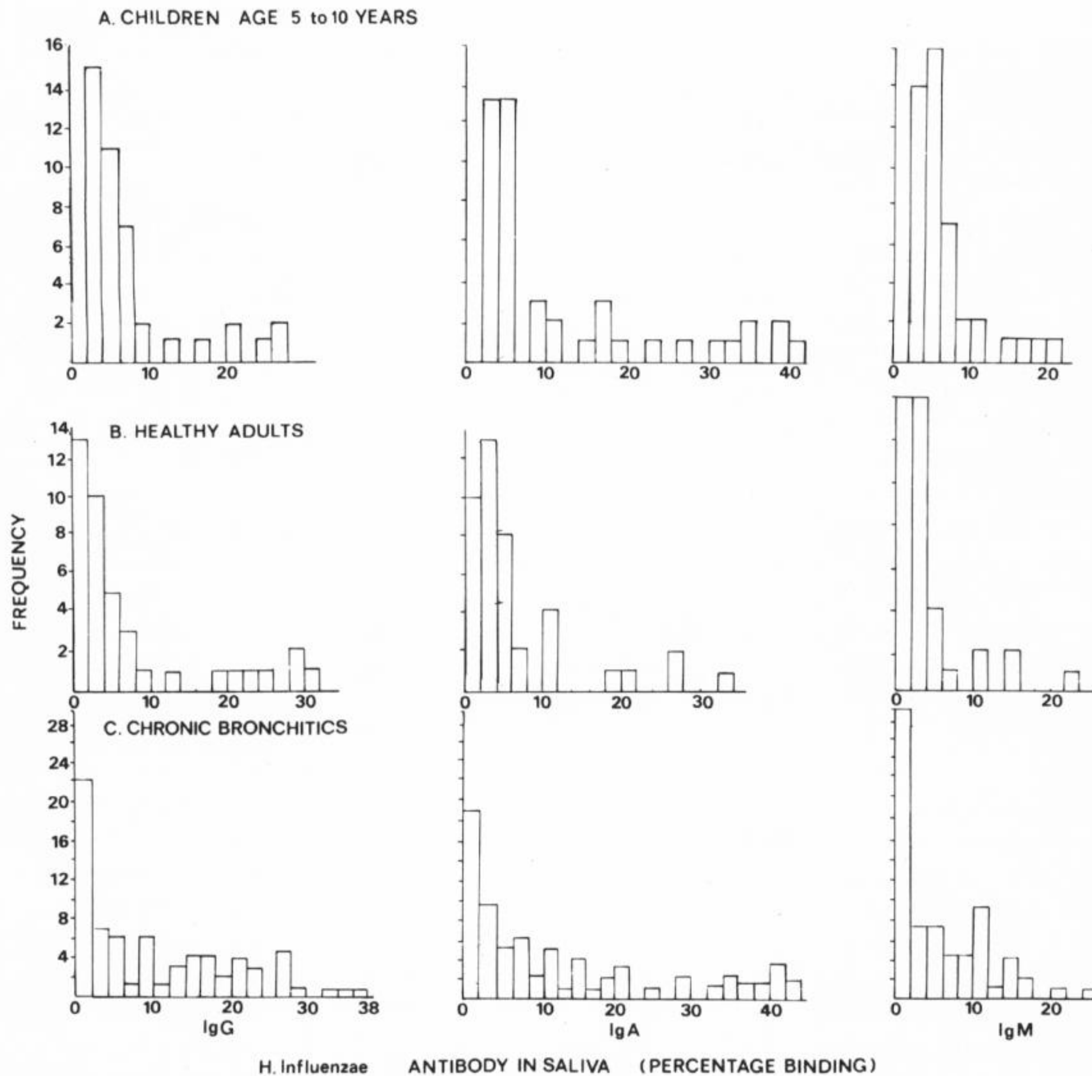

Fig. 5 Frequency distributions of *H. influenzae* antibody in the saliva of children aged 5-10 yr, normal adults and chronic bronchitics for the three immunoglobulin isotypes, IgG, IgA and IgM.

observed for IgM specific antibody, however, although the number of chronic bronchitics having higher levels of antibody tended to be greater compared with the children or adults this was not statistically significant at the 12% binding level. The occurrence of IgA specific antibody in the children and chronic bronchitics was consistently greater than that observed in adults for all levels of antibody ($P < 0.05$). At antibody levels equal to or greater than 12%, 32% of the children and 31% of the chronic bronchitics were positive compared with 12% of the adults. On the other hand, at antibody levels equal to or greater than 4%, 67% of the children and 62% of the chronic bronchitics were positive compared with 42% of the adults.

Clinical correlations

Subjects with chronic bronchitis were followed for 9 months after serum and saliva was taken for anti-*H. influenzae* antibody estimation. Clinical records of subjects with serum antibody binding of greater than or equal to 8% (15% of subjects tested) were compared with those with binding levels less than or equal to 3.5% (12.5%) of subjects tested. A significant difference ($P < 0.05$) in mortality but not respiratory function as analysed by the FEV_1 was observed. The infection rate in subjects with high serum antibody levels was approximately twice that of subjects with low serum antibody (Table 1). Salivary antibody levels did not correlate with serum levels, but did correlate with numbers of infection episodes in the 9 month period of follow-up.

DISCUSSION

We have developed a radioimmunoassay capable of detecting isotype specific antibody in both serum and mucosal secretions against the H_1/H_2 antigens of *H. influenzae*. The assay has been used to demonstrate different patterns of antibody in children, adults, and patients with chronic bronchitis. Previous assays have given limited information regarding the humoral immune response to *H. influenzae*. Some assays have used crude antigen which cross reacted with other gram-negative organisms.[2,3] The 'read out' of these assays has lacked sensitivity, being based on indices such as agglutination,[5] precipitation,[2,7,9,10] complement fixation[6] and immunofluorescence,[3,8] and at best have been semi-quantitative and not routinely class specific.

The advantages of the current assay are those of any radioimmunoassay. It is sensitive, quantitative, and easily modified to detect antibody isotype. It is technically easy to measure large numbers of small volume samples. The H_1/H_2 antigens were shown to be of large molecular weight, and unlabelled antigens could inhibit antibody binding for both serum and saliva, confirming specificity in this system. Previous studies have demonstrated specificity of H_1/H_2 antigens for *H. influenzae,*[4] though it has to be recognised that characterisation of somatic antigens of *H. influenzae* is at an early stage. For example, Clark has shown that some sera containing anti-*H. influenzae* antibody may not react with H_1/H_2 antigens and that various antigen prepaiations contain endotoxin.[19]

Use of this assay to quantitate class specific antibody in normal children and adults, as well as patients with chronic bronchitis, expands our knowledge of the host response to *H. influenzae*. Current understanding is scant, but still has been used to argue a case for a role for this organism in the pathogenesis of the complications of chronic bronchitis (reviewed by Tager and Speizer).[20] Semiquantitative studies demonstrating precipitating antibody in the serum of half the patients with chronic bronchitis compared with less than 16% of normal controls,[7,9,10] fail to clearly define the spectrum of host responses to *H. influenzae* in normal adults and chronic bronchitics. Variation of colonisation patterns with age (reviewed by Tager and Speizer),[20] has not been correlated with concomitant changes in immunity. Except for one study of class specific antibody to capsular antigen in nasal washings after systemic infection with *H. influenzae,* there is no data on the mucosal antibody response. Furthermore,[21] no examination of the local immune response in chronic bronchitis is available other than a limited study of mucosal T cell response.[22]

Against this sketchy background, our data demonstrates that as a group chronic bronchitics do not have a significantly different level of serum antibody of any class to *H. influenzae* specific somatic antigens. However, a significant 'tail' exists in the distribution of antibody in chronic bronchitic patients, with 20-25% having higher levels of binding of each isotype of antibody. Our analysis of the cumulative frequency of *H. influenzae* in the saliva of normal adults and chronic bronchitics demonstrates a significantly greater incidence of both high and low levels of antibody of IgG and IgA isotype in the chronic bronchitic population. The incidence of IgM antibody is also greater, but does not reach significance at higher levels of antibody binding.

Within the normal population we have also analysed the effect of age on antibody levels in saliva. The IgG and IgM antibody cumulative frequency profile was similar in both adults and children although a higher frequency of antibody of each isotype was found at low levels of antibody in children. The profile of IgA antibody in children differed significantly from the adult profile, with higher frequencies of antibody at all levels of antibody binding. The IgA profile of children resembled more that of chronic bronchitics than that of normal adults. The explanation of these age related differences is not obvious, but two possible explanations can be discussed. First, a reduced 'load' of bacteria with increasing age may be associated with lower levels of locally synthesised antibody. Second, antigen priming of mucosal sites is associated with a restriction of antibody response to subsequent immunisation,[23] possible due to an expansion of suppressor T lymphocytes within the mucosa.[24] Thus, levels of antibody within mucosal secretions are 'set' by the balance of helper and suppressor T lymphocytes with little or no change following variation in local antigen concentrations. The different profiles or incidence of antibody levels between children, normal adults and chronic bronchitics may well reflect differences between degrees of antigen stimulation and the balance between regulatory T lymphocytes.

Although the prime purpose of this study was to assess the antibody response to. *H. influenzae,* a limited prospective clinical appraisal was taken of the patients with chronic bronchitis. Patients with the highest levels of serum antibody to *H. influenzae* had a higher mortality and a higher incidence of clinical infection, but the groups did not have different spirometric patterns. It is not possible to determine from this data a causal effect for *H. influenzae* or the host response to infection with

this organism. Salivary antibody levels did not correlate closely with serum levels, but did correlate with the incidence of infection episodes, possibly reflecting local stimulus.

Longitudinal studies of normal subject, chronic bronchitics and patients with other chronic respiratory disorders with careful correlation of clinical, microbiological and immunological data, combined with estimation of antibody following local and systemic immunisation, may provide answers to some of the questions posed by these studies.

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