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Interleukin-2 Induced Activation of Natural Killer Cells in Rats and Mice : A Comparative Study.

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SUMMARY **Effects of interleukin-2 (IL-2) on the natural killer (NK) acivities of BALB/c mouse and Wistar rat spleen cells were compared. While mouse spleen cells cultured alone rapidly lost NK activity, co-culture with IL-2 resulted in a marked enhancement of NK activity. In contrast, the levels of NK activity of rat spleen cells cultured alone increased and remained high for 3 days and declined thereafter. Addition of human recombinant IL-2 or purified rat IL-2 did not influence the NK levels in rat spleen cell bulk cultures. Both IL-2 preparations were however biologically active as shown by their capacities to induced proliferation in rat spleen cells. Rat spleen cells suppressed the IL-2 activation of mouse spleen cells in a dose dependent manner, indicating a suppressor influence generated by rat spleen cells. Culture supernatants of rat spleen cells cultured with or without IL-2 for 3 or 5 days could also suppress the mouse spleen NK activation in response to IL-2. The suppressor activity could be concentrated on a 5K MW cut-off Amicon filter indicating that the molecular weight of the factor is more than 5000. These results indicate that a suppressor of IL-2 induced NK activation of mouse spleen cells is released by cultured rat spleen cells.**

Interleukin-2 (IL-2), a lymphokine produced by T-cells has profound effect on the natural killer lymphocytes of mouse and human origins. *In vitro* addition of IL-2 results in a marked enhancement of NK activity of mouse spleen cells [1-3] and human peripheral blood mononuclear cell preparations. [4] Mouse and human NK cells can proliferate in response to IL-2 and some investigators have established NK cell lines using IL-2 as a growth factor. [5,6] High levels of cytotoxic activity directed against NK resistant tumor target cells generated in response to IL-2 have been attributed to a catagory of killer cells termed lymphokine activated killer (LAK) cells. [7] The relationship of LAK cells to NK cells is not yet clear. *In vivo* administration of IL-2 is known to enhance the spleen NK activity in mice. [8,9] Capacity of IL-2 to induce NK activation and generation of LAK cells has formed the rationale for the use of IL-2 as an immunotherapeutic agent against cancers. [10] Although the influence of IL-2 on the NK system is well studied in mouse and human systems, little information in this regard is available for the rat system.

In the present study we examined the *in vitro* effect of IL-2 on the NK activity of rat spleen cells and compared it with the mouse system. Our results indicated that cultured rat spleen cells retained a high level of NK activity which could not be enhanced by addition of IL-2. The lack of IL-2 induced NK activation in the rat spleen could have been due to the generation of suppressor cells factors since IL-2 induced NK activation of mouse spleen cells was suppressed in the presence of rat spleen cells. Culture supernatants of rat spleen cells also inhibited the IL-2 activation of mouse spleen cells, indicating that a soluble suppressor factor may be secreted by rat spleen cells. The suppressor activity could be concentrated on a 5K MW cut-off Amicon membrane filter indicating that the molecular weight of the factor was more than 5000.

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MATERIALS AND METHODS

Animals.

BALB/c mice and Wistar rats were bred in the animal house at Jawaharlal Nehru University, New Delhi. Male animals between 6 to 12 weeks of age were used in all experiments.

Effector and target cells.

Spleens from rats or mice were dissected under sterile conditions and teased in Hank's BSS. Tissue debris was allowed to settle for a few minutes and then cells were collected, washed two times with BSS, and finally suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2×10^{-5} M 2-mercaptoethanol and 50 μg/ml gentamicin (complete medium). YAC, a mouse T-cell lymphoma cell line used as target cells was propagated in suspension cultures in complete medium. Target cells were counted and pelleted by centrifugation. To the cell pellets containing 10^6 cells, 0.1 mCi of ^{51}Cr as sodium chromate in normal saline (sp. activity = 0.1 Ci/mg chromium; Bhabha Atomic Research Centre, Trombay) and 10 μl of fetal bovine serum were added and the cell suspensions were incubated at 37° C for one hour. After the incubation period, labelled cells were washed three times and suspended in complete medium at a concentration of 10^5 cells/ml.

Chromium release assay of cytotoxicity

The required number of effector cells and chromium-labelled target cells were deposited in round bottom wells of 96-well microtest plates (0.2 ml/well) and assay plates were centrifuged at 60 × g for 5 minutes. Assay plates were incubated at 37° C in a humidified atmosphere of 5% CO_2 and 95% air for 4 hours. The amount of radioactive chromium released in each assay well was estimated by counting 0.1 ml of culture supernatant in an LKB gamma counter. Spontaneous release of ^{51}Cr was determined by incubating target cells in the absence of effector cells and maximum release of ^{51}Cr was determined by water lysis of labelled cells. Percent target lysis in each well was calculated as described before.[11]

Interleukin-2 activation

Effector cells were activated by IL-2 either in 96-well microtest plates or in tissue culture flasks. The human recombinant IL-2 preparation used in all experiments was a gift from Hoffmann La Roche, Nutley, NJ, USA. A highly purified preparation of rat IL-2 obtained by isoelectric focusing was made in our laboratory as described before.[12] Exact protocols for activation are described in the legends to the figures and tables. IL-2 preparations were assayed on the IL-2 dependent CTLL line as previously described.[13]

RESULTS

Effect of human recombinant interleukin-2 (IL-2) on the anti-YAC cytotoxic activity of cultured mouse and rat spleen cells

Activation of mouse spleen natural killer activity by IL-2 is well documented in the literature.[1-3] In order to examine the effect of IL-2 in the rat system, rat spleen cells and control mouse spleen cells, were cultured with or without IL-2 (100 U/ml) for up to 7 days. At different times, the cells were harvested, washed and used as effector cells in a standard 4 hour chromium-release assay using YAC target cells. Results indicated that the basal NK activity was higher in rat spleen cells than in mouse spleen cells (Fig. 1, day-0). Mouse spleen cells cultured with IL-2 acquired significantly higher cytotoxic activity on day 1. This

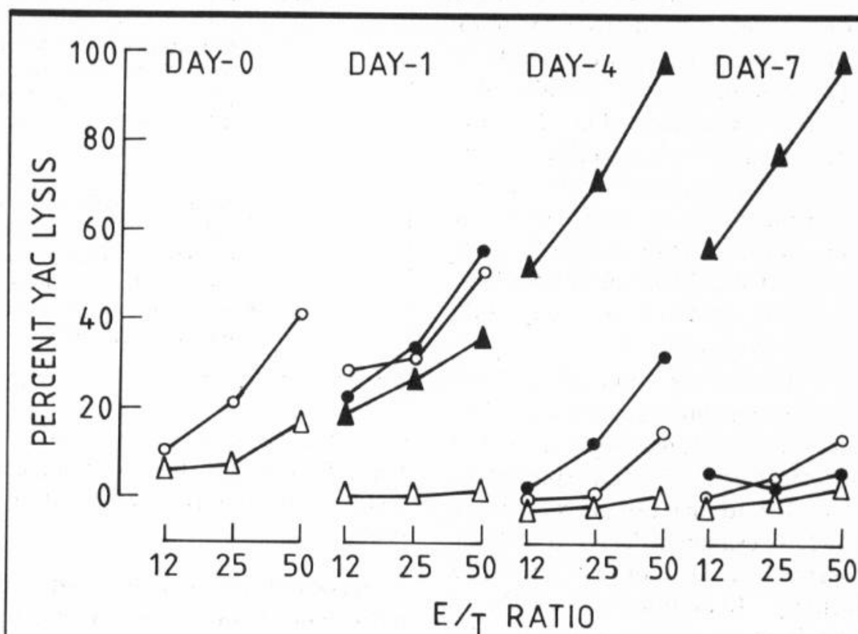

Fig. 1 Kinetics of the effect of IL–2 on the NK activities of mouse and rat spleen cells. Mouse and rat spleen cells were cultured (5×10^6 cells/ml in complete medium) with or without 1,000 U/ml of human recombinant IL–2. At different times, cells were washed and used as effector cells in a cytotoxicity assay using YAC tumor cells as the target. Lytic activities of fresh and cultured spleen cells were determined at three E/T ratios. Results for control mouse (△—△) and rat (○—○) spleen and for IL–2 activated mouse (▲—▲) spleen rat (●—●) spleen cells are shown. Each point represents a mean of three replicate wells.

increased further by days 4 and 7 of the culture. Control cultured mouse spleen cells did not exhibit any cytolytic activity. In contrast, control rat spleen cells retained significant cytolytic activity up to day 4 of culture. In several similar experiments, we observed that the levels of NK activity in control rat spleen cells increased initially, peaked on day 1 or 2 of culture and declined thereafter. Rat spleen cells cultured with IL-2 did not acquire significantly higher levels of cytolytic activity than controls, especially when compared to the activation seen with mouse spleen cells. However, it should be noted that the NK levels for treated cells on day 4 were lower than the NK levels in the control rat spleen cells on day one.

In previous experiments IL-2 was used at a single dose of 100 U/ml. It was possible that this dose was not sufficient/optimal for the activation of rat NK cells. Attempts were therefore made to activate rat spleen cells with a wider range of IL-2 concentrations. Results of a typical experiment (Fig. 2) show that the mouse spleen cells were activated in a dose-dependent manner after three days of culture with concentrations of IL-2 ranging from 60 to 1000 U/ml. Rat spleen cells however were not activated at any of the tested doses of IL-2.

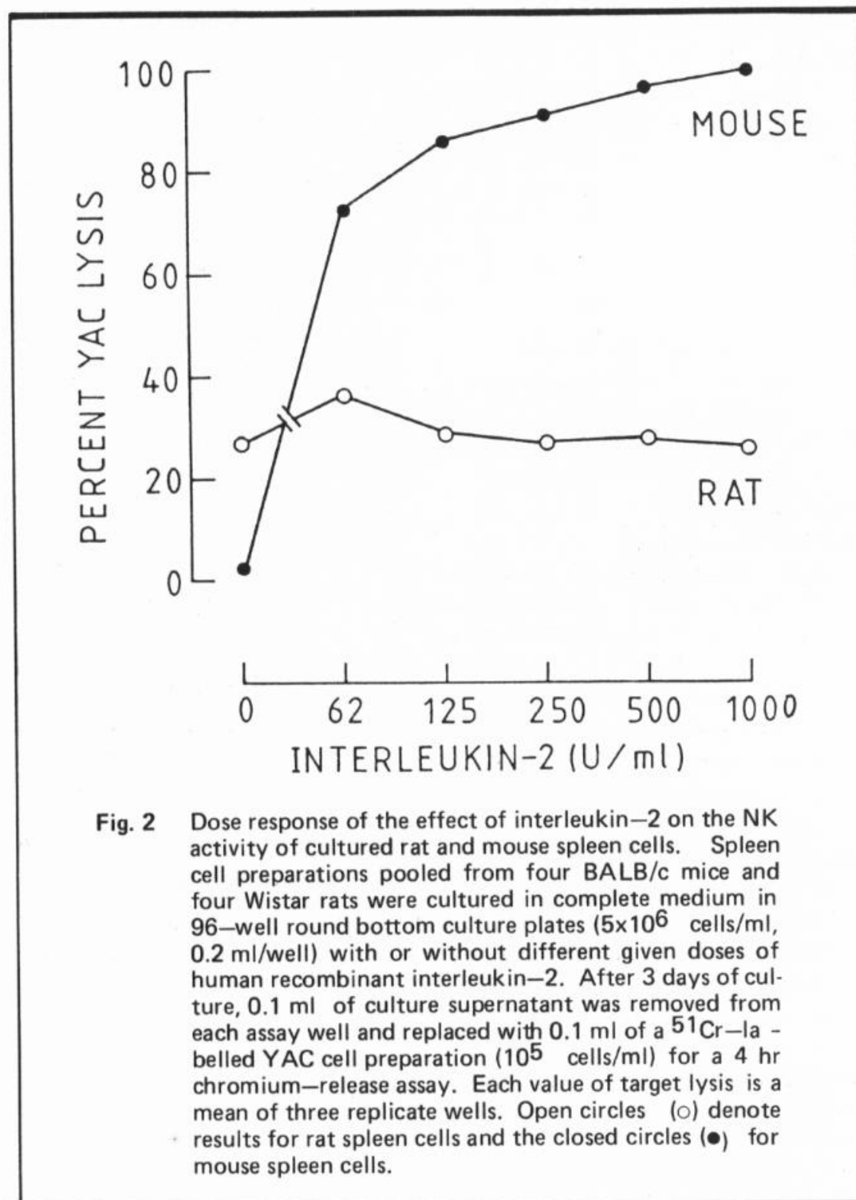

Fig. 2 Dose response of the effect of interleukin–2 on the NK activity of cultured rat and mouse spleen cells. Spleen cell preparations pooled from four BALB/c mice and four Wistar rats were cultured in complete medium in 96–well round bottom culture plates (5×10^6 cells/ml, 0.2 ml/well) with or without different given doses of human recombinant interleukin–2. After 3 days of culture, 0.1 ml of culture supernatant was removed from each assay well and replaced with 0.1 ml of a ^{51}Cr–labelled YAC cell preparation (10^5 cells/ml) for a 4 hr chromium–release assay. Each value of target lysis is a mean of three replicate wells. Open circles (○) denote results for rat spleen cells and the closed circles (●) for mouse spleen cells.

Human recombinant IL-2 was used in experiments described above. In order to examine whether the lack of effect of IL-2 on rat splenocytes was due to the use of xenogeneic IL-2, we examined the effect of highly purified rat IL-2 on rat spleen NK activity. In addition to its effect on the level of cytolytic activity, the effect of IL-2 preparations on the rate of proliferaton of rat spleen cells was also examined. Results of these experiments (Table 1) indicate that both human recombinant IL-2 and rat IL-2 preparations enhanced the proliferative activity of rat spleen cells without influencing the level of cytolytic activity. Therefore, the lack of effect of IL-2 on rat spleen NK cells was not due to biological inertness of the IL-2 preparations used.

Suppression of IL-2 induced NK activation of mouse spleen cells by rat spleen cells

Since the cytotoxicity levels of rat spleen cells could not be significantly augmented by IL-2, the possibility of a suppressor influence was considered. However, detection of suppression was difficult within the rat system, since no IL-2 induced NK activation was demonstrable. Good NK activation in the murine system prompted us to use it to detect suppression. We compared the NK activation in rat and mouse spleen cells alone with the activation of a 1:1 mixture of rat and mouse spleen cells. A comparison of cytolytic activation in these individual and mixed cell preparations (Table 2) clearly shows (a) that the mouse spleen cells were activated and that the activation increased with culture duration, (b) that rat spleen cells had a relatively higher basal NK activity but failed to be activated by IL-2, and (c) that the mixture

Table 1. Effect of interleukin–2 on the cytotoxic activity and DNA synthesis activity of rat spleen cells[a]

Expt.	Culture duration (days)	Cytotoxic activity[b]			DNA synthesis activity[c]		
		Control	+HR IL–2	+Rat IL–2	Control	+HR IL–2	+Rat IL–2
1	3	4.9 ± 0.6	9.9 ± 1.3	9.5 ± 1.8	5,794 ± 141	37,748 ± 1,837	29,629 ± 7,197
	6	2.5 ± 2.2	4.6 ± 0.3	4.8 ± 0.7	4,474 ± 905	38,450 ± 1,357	40,034 ± 8,284
2	3	5.6 ± 0.7	7.4 ± 0.3	10.1 ± 2.1	9,977 ± 839	61,166 ± 1,633	57,812 ± 6,904
	6	2.0 ± 0.7	5.6 ± 0.5	6.5 ± 0.3	4,565 ± 576	29,792 ± 177	29,382 ± 2,979
3	3	8.4 ± 1.5	7.1 ± 1.5	8.7 ± 1.5	–	–	–
	6	3.0 ± 0.2	6.7 ± 1.0	4.5 ± 0.1	3,136 ± 258	31,594 ± 2,194	14,211 ± 1,897

[a]Rat spleen cells (0.5×10^6 in 0.2 ml complete medium) in 96 well plates (round bottom for cytotoxicity assay, flat bottom for thymidine incorporation assay) were cultured for 3 or 6 days with or without human recombinant IL–2 (100 U/ml) or rat IL–2 (100 U/ml).

[b]For the assay of cytotoxic activity, 0.1 ml supernantant from ^{51}Cr–labelled culture wells was removed without disturbing the cell pellets and replaced with YAC cells (10^4 cells in 0.1 ml medium). Plates were centrifuged at 500 RPM for 3 minutes. A 4 hr chromium–release assay of cytotoxicity was done as described in the Methods. Each value of YAC lysis is a mean ± SD of three replicate wells.

[c]After the given culture duration, an 18 hr pulse of (^3H)–Thymidine (0.5 μCi in 10 μl/well) was given. Each value represents a mean ± SD of CPM value obtained from 3 replicate wells.

Table 2. Suppression of IL–2 induced NK activation in mixture and individual cultures of rat and mouse spleen cells[a]

Culture duration	E/T ratio	Percent YAC Lysis					
		Mouse spleen cells		Rat spleen cells		1 : 1 mixture	
		Control	+IL–2	Control	+IL–2	Control	+IL–2
3 days	50	0.4	77.9	28.6	23.6	2.8	34.8
	25	0.5	53.5	15.4	15.9	0.9	20.2
	12	0.7	24.1	8.2	2.8	0.4	11.0
[b](% cell recovery		73.7	113.1	51.9	46.2	60.6	71.9)
5 days	50	3.7	81.2	10.8	2.9	0.4	10.6
	25	3.5	72.0	4.2	2.0	0.4	5.8
	12	2.5	61.1	3.1	0.3	0.7	4.3
[b](% cell recovery		75.6	79.4	39.4	31.9	63.1	53.7)

[a]Mouse spleen cells, rat spleen cells and a 1 : 1 mixture of both were cultured in complete medium (5×10^6/ml ± 100 U/ml of IL–2). After 3 or 4 days of culture, cells were washed and assayed for cytotoxic activity. Each value of percent target lysis is a mean of three replicate assay wells.

[b]Value of viable cells recovered from cultures, as percentage of initial input, are shown in parentheses.

of rat and mouse spleen cells could be activated by IL-2, but that the activation was poor when compared to the activation of mouse spleen cells alone. The suppressive effect of rat spleen cells could be observed on day 3 and was particularly marked on day 5. Percent recovery of original viable cells at the end of culture is also shown in Table 2. In general, the recovery from mixed cultures was approximately an average of the recovery for the individual cultures.

Our results further indicated that the suppressive effect of rat spleen cells on IL-2 induced activation of mouse spleen cells was dose dependent (Fig. 3). Significant suppression could be observed even at a rat : mouse spleen cell ratio of

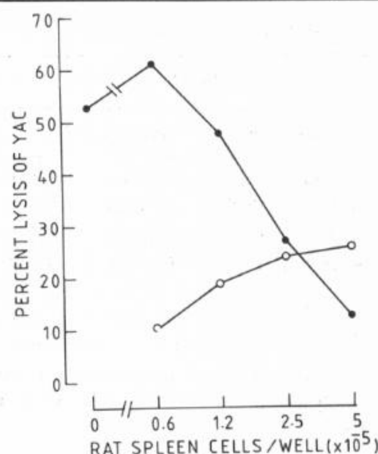

Fig. 3 Dose dependent suppression of IL–2 induced activation of mouse spleen NK activity by rat spleen cells. Mouse splenocytes were cultured in round bottom wells of microtest plates (5×10^5 cells/well in 0.2 ml complete medium containing IL–2 100 U/ml) with or without different given concentrations of rat spleen cells for 3 days. At the end of the culture, 0.1 ml of the culture supernatant was removed from each well and replaced with 0.1 ml of labelled YAC cells suspension for a 4 hr chromium-release cytotoxicity assay. Data for mouse spleen cells activated alone or in the presence of different concentrations of rat spleen cells (●) and for rat spleen cells cultured alone with IL–2 (○) are given. Each value of target lysis is a mean of three replicate assay wells.

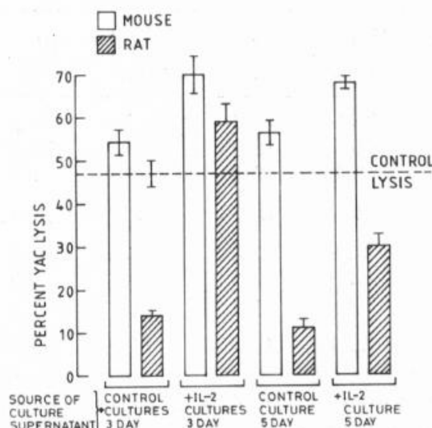

Fig. 4 Effects of culture supernatants of mouse and rat spleen cells on IL–2 induced NK activation of mouse spleen cells. Mouse and rat spleen cells were cultured ± IL–2 (100 U/ml) for 3 and 5 days (5×10^6 cells/ml in complete medium) and culture supernatants were collected by centrifugation. These supernatants were filtered (0.45 μ) and frozen at −70°C until used. For testing the effect of these supernatants, mouse spleen cells were cultured in 96–well round–bottom microculture plates (2.5×10^5 cells in 0.2 ml medium containing 100 U/ml of IL–2 in each assay well). Various test culture supernatants were included in these cultures at 80% v/v final concentration. After 3 days of culture, 0.1 ml of supernatant from each assay well was carefully removed and replaced with 0.1 ml of labelled YAC cells (10^5 cells/ml) for a 4 hr cytotoxicity assay. Each value in the figure represents mean ±SD of three replicate observations.

1:8 and the suppression was almost complete at a 1:1 ratio of the two types of effector cells (Fig. 3). Mouse spleen cells added as a control for rat spleen cells did not suppress the NK activation phenomenon (results not shown).

Evidence for a suppressor factor released by rat spleen cells

Since rat spleen cells could inhibit IL-2 induced augmentation of mouse spleen cell NK activity, we determined whether this suppressor action was mediated by a soluble factor(s). This was done by testing the effect of rat spleen cell culture supernatants on the IL-2 induced NK activation of mouse spleen cells. Rat spleen cells were cultured with or without IL-2. After 3 or 5 days, the culture supernatants were collected for testing. Mouse spleen culture supernatants prepared in an identical way were used as controls. Mouse spleen cells were activated for three days by IL-2 in the presence or absence of various types of test supernatants. Results of a representative experiment are given in Fig. 4. While none of the mouse supernatants suppressed NK activation, control rat supernatants were significantly suppressive. Three-day rat spleen + IL-2 supernatant was not suppressive while five-day rat spleen + IL-2 supernatant was suppressive. It should be noted that supernatants prepared from IL-2 treated cultures had residual IL-2 which added to the IL-2 used in the suppression assay. These results indicated that supernatants of cells cultured alone for 3 or 5 days or with IL-2 for 5 days contained a suppressor factor(s) which interfered with IL-2 induced NK activation of mouse spleen cells. In order to estimate the molecular weight of this suppressor factor(s), supernatants of 5 day rat spleen cell cultures (R5) were concentrated five-fold using a 5000 MW cut-off ultrafiltration membrane. Results (Fig. 5) indicated that the suppressive

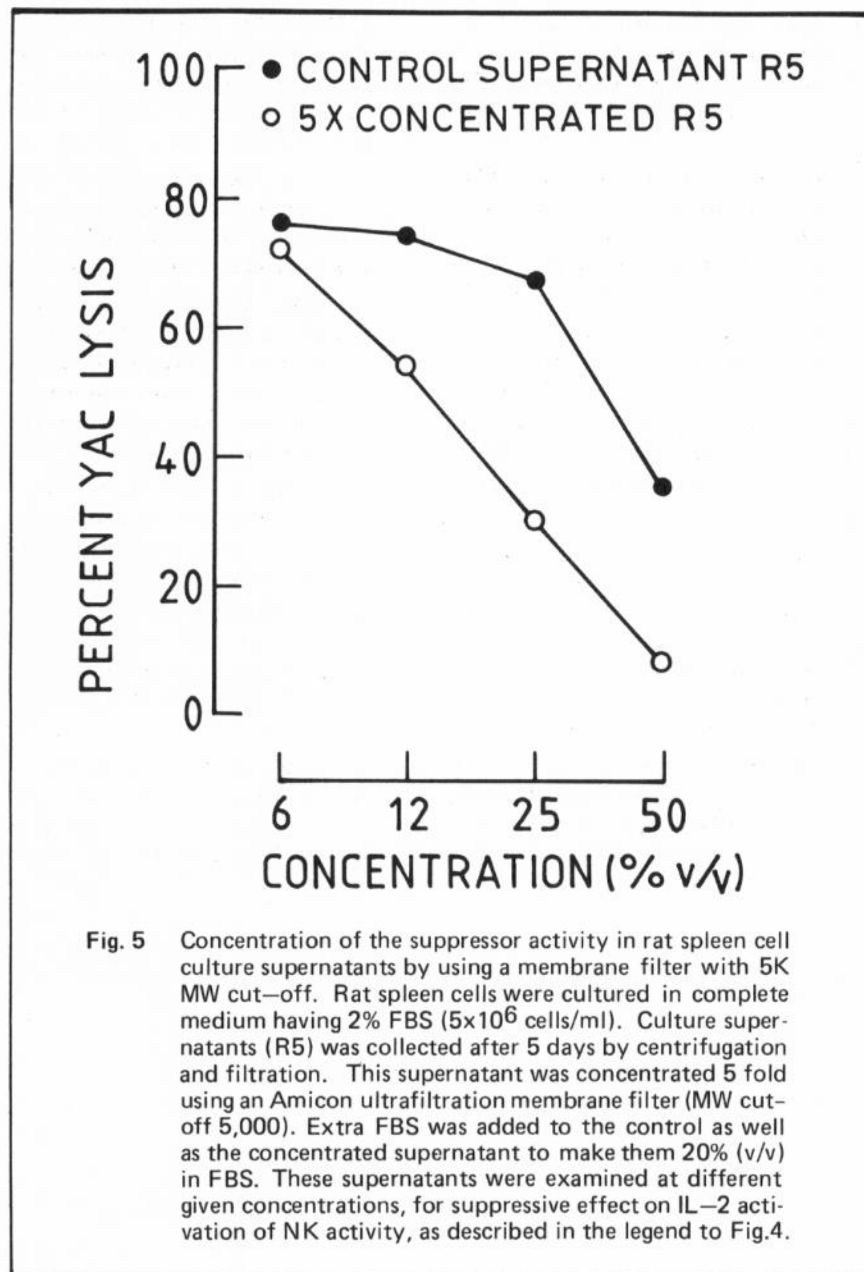

Fig. 5 Concentration of the suppressor activity in rat spleen cell culture supernatants by using a membrane filter with 5K MW cut–off. Rat spleen cells were cultured in complete medium having 2% FBS (5×10^6 cells/ml). Culture supernatants (R5) was collected after 5 days by centrifugation and filtration. This supernatant was concentrated 5 fold using an Amicon ultrafiltration membrane filter (MW cut-off 5,000). Extra FBS was added to the control as well as the concentrated supernatant to make them 20% (v/v) in FBS. These supernatants were examined at different given concentrations, for suppressive effect on IL–2 activation of NK activity, as described in the legend to Fig.4.

activity of the supernatant was concentrated and that the suppressor factor(s) had a MW higher than 5 K.

DISCUSSION

In the present study we have compared the effect of IL-2 on the level of NK activity of rat and mouse spleen cells. Two major differences were found between the rat and mouse systems. First, mouse spleen cells rapidly lose their NK activity in culture, while rat spleen cells retain a substantial basal NK cell cytotoxic activity up to the fourth day of culture. Second, IL-2 is known to be a potent stimulator of NK cell activity in human and mouse systems, but we could not demonstrate a similar effect of IL-2 on rat spleen cells. Nor did purified rat IL-2 influence the NK-activity of rat spleen cells. It should be noted that the proliferative activity of rat spleen cells was augmented by both human recombinant IL-2 and rat IL-2. Clearly, therefore, the lack of effect of IL-2 on rat NK cells was not a result of inactive IL-2 preparations. Further, we have tested a wide range of IL-2 doses and exposure times, but still did not observe an augmentation of NK activity. Although we used Wistar rats in our studies, similar results were obtained by using Sprague Dawley rats (our unpublished data). However, the possibility of strain dependence on the phenomenon of IL-2 induced NK activation in the rat system must be left open.

Lack of IL-2 activation of rat NK activity could either be due to an inherent inability of rat NK cells to respond to IL-2 or due to the generation of some suppressor cell/factor which interferes with the activation process. Experiments involving the activation of mixed spleen cell preparations indicated that the mouse spleen cells could not be activated by IL-2 in the presence of rat spleen cells. A mixture of rat and mouse spleen cells would constitute a xenogeneic mixed lymphocyte reaction (MLR) and the suppression of IL-2 induced NK activation of murine spleen cells could be related to some suppressor influence generated in MLR. By contrast, allogenic murine MLR results in augmentation of NK activity rather than suppression.[14] Since spleen cell culture supernatants alone could suppress NK activation of mouse spleen cells in response to IL-2, the suppressor influence was not a consequence of cell contact. The suppressor factor appeared to be released by the spleen cells in a constitutive manner and the presence of IL-2 was not needed for its induction. From the results of our experiments using Amicon ultrafiltration membranes, the suppressor factor appeared to have a molecular weight greater than 5 K.

In conclusion, we may hypothesise that the suppressor factor in rat spleen cell supernatants is responsible for their lack of IL-2 induced NK activation. We have

not provided direct evidence for this hypothesis because experiments to test it would require neutralization of the factor in rat spleen cell cultures. Further characterization of the suppressor factor is required before this can be done.

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