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Potentiation of natural killer cell activity of human lymphocytes *in vitro*: the participation of interferon in stimulation with *Staphylococcus aureus* Cowan I bacteria but not with Protein A

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Summary. In the previous paper we reported that human natural killer (NK) cell activity was augmented greatly by preincubation with *Staphylococcus aureus* Cowan I bacteria (SpA CoI) or its Protein A. We examined here whether the augmentation with these stimulants is ascribable to the direct activation of NK cells or mediated by some soluble factors produced by the stimulants. It was found that a significant amount of interferon (IFN) was produced by the SpA CoI-stimulation but not by the Protein A-stimulation, although the latter usually induced augmentation of NK-cell activity not less than SpA CoI-stimulation. IFN produced by SpA CoI was considered to belong to α-type IFN, because it was stable at pH 2·0 and could be neutralized effectively by anti-IFNα antibody. Kinetics of NK-cell activation by SpA CoI (but not by Protein A) were very similar to those by IFNα. Furthermore, augmentation of NK-cell activity with SpA CoI-stimulated supernatant was inhibited almost completely by diluted anti-IFNα antibody, whereas augmentation with Protein A-stimulated supernatant could not be abolished by the same treatment. It was, therefore, suggested that augmentation of NK-cell activity with SpA CoI might be ascribable in most part to the IFN induced, whereas Protein A can stimulate NK or T cells directly or soluble factors other than IFN might work as well.

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INTRODUCTION

Lymphocytes from normal animals and humans are capable of killing a variety of tumour and leukaemic cell lines spontaneously: this cytotoxicity is mediated by natural killer (NK) cells. NK cells have attracted in recent years considerable attention because of their potential role in the prevention of tumour and leukaemia development (Takasugi, Mickey & Terasaki, 1973; Herberman, Nunn & Lavrin, 1975; Haller, Hasson, Kiessling & Wigzell, 1977; Collins, Patek & Cohn, 1981). Agents or manipulations raising NK-cell activity *in vivo* and *in vitro* are therefore of particular importance and have been largely extended. Immunostimulants of bacterial origin, BCG and *Corynebacterium parvum* (Wolfe, Tracey & Henney, 1977; Macfarlan, Ceredig & White, 1979), chemical agents such as poly I:C, tilorone, statolon (Welsh, 1978) as well as many viruses and tumour cells (Djeu, Heinbaugh, Holden & Herberman, 1979a; Trinchieri &

Santoli, 1978) could boost NK-cell activity *in vivo* and *in vitro*. Most of these immunostimulants are known also to the inducers of interferon (IFN), so that the augmentation of NK-cell activity may be ascribable to the induced IFN (Gidlund, Örn, Wigzell, Senik & Gresser, 1978; Trinchieri, Santoli, Dee & Knowles, 1978; Zarling, Eskra, Borden, Horoszewicz & Carter, 1979; Djeu, Huang & Herberman, 1980). We reported in the previous paper that *Staphylococcus aureus* Cowan I (SpA CoI) bacteria and its Protein A are potent activators of human NK-cell activity (Kasahara, Harada, Shioiri-Nakano, Imai & Sano, 1981). In this paper we show that the IFN plays a major role in the augmentation of NK-cell activity by the SpA CoI stimulant, but might only play a minor role in, the activity of soluble Protein A.

MATERIALS AND METHODS

Cell separation

Cell separation procedures were described elsewhere (Kasahara, Kin, Itoh, Kawai, Morita & Shioiri-Nakano, 1980; Kasahara *et al.*, 1981). Peripheral blood lymphocytes from normal donors were separated with Ficoll-Urografin ($\rho = 1{\cdot}078$) density sedimentation, centrifuged at 2200 r.p.m., for 20 min. These unfractionated cells were further separated into T, B and null cells, if necessary. In brief, cells were first applied on a nylon fibre column (NC), incubated at 37° for 45 min and eluted with warmed Eagles' minimum essential medium (MEM, Nissui Seiyaku Co., Tokyo) supplemented with 7% foetal calf serum (FCS, Flow Laboratories, Dainippon Seiyaku, Osaka). T cells were separated from the NC-passed fraction by rosetting with 2-aminoethylisothiouronium bromide hydrobromide (AET)-treated sheep red blood cells (E). Null cells were separated from the NC-passed fraction by the removal of E-rosette-forming and surface immunoglobulin (SmIg)-rosette-forming cells (Kasahara *et al.*, 1981). B cells (containing 2%–9% monocytes and macrophages) were enriched from the NC-retained fraction by the removal of E-rosette-forming cells, followed by the enrichment of SmIg-rosette-forming cells. Cells thus obtained were usually treated with distilled water for 20 s to remove contaminating red blood cells. In some experiments, non-adherent cells were separated from the adherent cell population by non-adherence onto plastic dishes (Falcon number 5220) for at least 2 hr. Adherent cells were recovered by gentle scratching with a rubber policeman.

Assay of NK-cell activity

An erythromyeloid cell line, K-562, was grown and maintained in RPMI 1640 medium (Nissui Seiyaku) containing 7% FCS. Cells were labelled with 100 μCi of $Na_2{}^{51}CrO_4$ (30–70 mCi/mg, Japan Isotope Association) by incubation at 37° for 90 min. After four washings with medium, 1×10^5 K-562 target cells in 0·1 ml were mixed with serially diluted effector lymphocytes in 0·1 ml in a 96-well microculture plate (Linbro, number 76-002-05, Hamden, Conn). After 4·5 hr incubation, plates were centrifuged and 0·1 ml of the supernate was removed and measured by Aloka gamma counter. The specific ^{51}Cr release was calculated as described previously (Kasahara *et al.*, 1981).

Determination of antiviral (IFN) activity

Antiviral activity in the cultured supernatants was determined by three different laboratories where each different method was employed. A plaque reduction (performed by Dr Sugiura, National Institute of Public Health, Tokyo) was performed using mono-

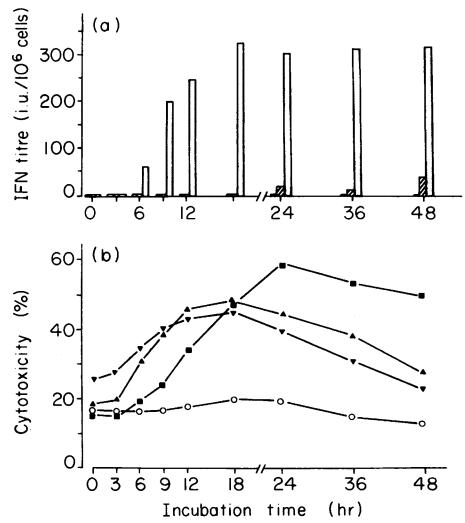

Figure 1. Kinetic study of NK-cell activation and IFN production. Cells/ml (1×10^6) of unfractionated cells were incubated with or without stimulants for the period indicated. Incubation was stopped at each time and one was immediately assayed for its NK-cell activity; the other was spun down and the supernatant was stocked for the assay of IFN activity. (a) IFN titre was determined by the methods described in Materials and Methods. (■) Protein A (10 μg/ml); (□) SpA CoI (0·005%). (b) NK-cell activity: (■——■) Protein A; (▲——▲) SpA CoI; (▼——▼) IFNα (1000 i.u./ml); E:T ratio was 10:1.

layers of human conjunctive cell line, I-5C-4 (Sugiura & Kilbourne, 1965) and vesicular stomatitis virus (VSV, an Indiana strain) as a challenge virus. Inhibition of cytopathic effect (CPE) was kindly done by Dr N. Minato (Department of Medicine, Jichi Medical School) using FL cells and VSV as a challenge (Haung, Donahoe, Gordon & Dresser, 1971). IFN assay by inhibition of viral RNA synthesis was routinely done in the Department of Immunology, Jichi Medical School, as the methods described by Kawade, Matsuzawa, Yamamoto, Tsukui & Iwakuva (1976). In brief, human foreskin fibroblast cells (Fs-7) suspended in 10% FCS-MEM were dispensed in 0·1 ml aliquots into microculture plates (96-wells, Linbro) and incubated at 37° overnight so that they form a confluent cell sheet. Serial dilutions of IFN preparations (50 μl) were added and left at 37° for at least 8 hr. The fluid is replaced by 0·05 ml of MEM containing VSV (multiplicity of 5–25 PFU/cell) in the presence of 0·25 μg actinomycin D. After 1·5 hr incubation at 37°, 0·5 μCi of [^{3}H]-uridine was added, followed by further 8 hr incubation. Fluid was then aspirated, the cell sheets were washed once and stood in cold 5% trichloroacetic acid, aspirated and then washed once more with the acid, twice with ethanol. The content of each well is dissolved in 0·15 ml of 2% sodium dodecyl sulphate and transferred to a filter paper disk (24 mm diameter), of which radioactivity was measured after drying. IFN titre was expressed as the International Units (I.U.) by comparing the potencies of a standard International Reference, IFN-α (N.I.H. human reference IFN G-023-901-527).

Acid stability of IFN

The samples were adjusted to pH 2·0 by the dropwise addition of 5 N HCl (7·5 μl/ml), left at 4° for 20 hr. After the pH was then brought back to pH 7·2 by the addition of 5 N NaOH, antiviral activity was measured.

Preparation of anti-IFNα antiserum

Human IFN was produced in human peripheral blood leucocytes infected with 100 HA unit/ml of Sendai virus (HVJ) for 20 hr. The IFN preparation was applied on an adsorbent column of anti-IFNα antibody (Namalva)-conjugated Sepharose 4B and eluted with 4 M NaI. The IFNα was purified up to 10^7 i.u./mg protein by this step. A sheep was immunized subcutaneously with 3×10^7 i.u. of the IFNα emulsified in Freund's complete adjuvant (Sigma), monthly for 6 months. Antiserum was absorbed with homogenized human leucocytes, serum and HVJ virus which were immobilized to Sepharose 4B. No cross-reactivity was observed between anti-IFNα serum and human lymphocytes, serum or HVJ virus by the Ouchterlony double diffusion technique. The anti-IFNα serum was able to neutralize 10 i.u. of IFNα to less than 1 i.u. at a 1:50,000 dilution.

Potentiation of NK-cell activity by the cultured supernatant

Culture supernatants were collected after cells were incubated with stimulants for 24 hr, filtered through millipore membrane (0·22 μm) and their potentiating activity was tested. The supernatants were added to the fresh lymphocyte cultures from autologous or allogeneic donors usually at a final concentration of 50%. Cytotoxic activity was determined after a further 24 hr incubation.

Stimulants

Staphylococcus aureus Cowan I strain bacteria were killed by 0·5% formaldehyde and treated with heat at 80°, for 5 min. Staphylococcal Protein A (Pharmacia Fine Chemicals, Uppsala, Sweden), phytohaemagglutinin (PHA, Difco Laboratories, Detroit, U.S.A.) and concanavalin A (Con A, E-Y Laboratories, Maruzen Oil, Tokyo) were purchased commercially.

RESULTS

Potentiation of cytotoxic activity of human lymphocytes by Protein A and SpA CoI bacteria

We have previously reported that preincubation of lymphocytes with Protein A or SpA CoI bacteria augmented cytotoxic activity against K-562 target cells greatly (Kasahara *et al.*, 1981). Cytotoxicity against K-562 target was mainly carried out by E-rosette negative, SmIg-rosette negative, (and Fc-receptor positive) null cells, i.e. NK cells and the participation of T and B cells was minimal, if any (Table 1).

Correlation between augmentation of NK activity and IFN production

In order to examine whether the augmented NK activity was ascribable to the direct activation of NK cells or mediated by some soluble factors (in particular IFN) produced by the stimulants, IFN activity in the culture supernatant was determined. As shown in

Table 1. Correlation between augmentation of NK activity and IFN production

Cell population*	Stimulants		Specific ^{51}Cr release at E:T ratio 10:1†	IFN titre i.u./10^6 cells‡
Unfractionated	Blank		14·8±3·2	<5
	Protein A	10 μg/ml	48·1±4·4	<5
	SpA CoI	0·005%	45·5±6·3	130
	PHA	10 μg/ml	38·0±2·2	<5
	HVJ	100 HA/ml	NT	>1000
NC-passed, E^+ (T cells)	Blank		8·5±1·6	<5
	Protein A	10 μg/ml	10·7±2·8	9
	SpA CoI	0·005%	8·9±2·2	6
	PHA	10 μg/ml	9·2±1·6	<5
	HVJ	100 HA/ml	NT	<5
NC-passed, E^- $SmIg^-$(null cells)	Blank		30·7±5·8	12
	Protein A	10 μg/ml	59·5±7·2	16
	SpA CoI	0·005%	60·5±11·2	500
	PHA	10 μg/ml	53·2±6·8	16
	HVJ	100 HA/ml	NT	500
NC-retained, E^-(B+Mφ)	Blank		12·5±2·1	28
	Protein A	10 μg/ml	13·7±2·4	<5
	SpA CoI	0·005%	29·3±3·6	250
	PHA	10 μg/ml	15·4±1·9	9
	HVJ	100 HA/ml	NT	>1000

* Peripheral blood lymphocytes (number 064, 067–071) were separated into NC-passed, E^+ (T), NC-passed, E^-, $SmIg^-$ (null) and NC-retained, E^-(B+Mφ) cells. These cell populations were adjusted to 1×10^6 cells/ml and incubated for 24 hr with or without stimulants.

† NK activity against ^{51}Cr-labelled K-562 target was measured after 4·5 hr incubation.

‡ Antiviral activity in the 24 hr cultured supernatant was determined by the inhibition of viral RNA synthesis as described in Materials and Methods.

Table 1, a significant amount of IFN was detected in the supernatant obtained with SpA CoI but not with Protein A, although no less augmentation of NK activity was obtained by the stimulation with Protein A. IFN production with the SpA-CoI stimulated culture was highest in null cell (500 i.u./10^6 cells) and B (+Mφ) cell fractions (250 i.u.), but no significant IFN was induced in the T-cell fraction (6 i.u.). These data suggest that augmentation of NK activity by SpA CoI and Protein A was mediated by different pathways and NK activation by the SpA CoI-stimulation was ascribable possibly to the mediation of IFN produced in the culture.

Determination of IFN production by the three distinct methods

IFN activity described as above was measured by the inhibition of viral RNA synthesis. Antiviral activity was determined by other techniques, plaque reduction and inhibition of CPE in order to confirm further the production of IFN. Similar results were, however, observed by these methods (Table 2), namely, a significant amount of IFN was produced by the SpA CoI-stimulation, while only a minimal amount of IFN was detectable by the Protein A stimulation. In further experiment, antiviral activity was determined routinely by the method of inhibition of viral RNA synthesis.

Type of IFN produced by the SpA CoI-stimulation

The nature of IFN produced by the SpA CoI-stimulation was examined as regards the acid stability and the neutralization by the anti-IFN antibody. As shown in Table 3, no significant reduction of antiviral activity in

Table 2. Induction of IFN by the stimulation of SpA CoI bacteria but not by Protein A

Cell population	Stimulant	IFN titre (i.u./10^6 cells/ml)* 50% Plaque reduction Exp. 1 (no. 074)	50% Plaque reduction Exp. 2 (no. 079)	Inhibition of CPE† Exp. 3 (no. 089)	Inhibition of uridine uptake Exp. 4 (no. 080)	Inhibition of uridine uptake Exp. 5 (no. 084)
Unfractionated	Blank	<2	<2	<2	<2	<2
	Protein A	16	<2	2	2·5	14
	SpA CoI	128	32	128	83	200
NC-passed	Blank	<2			<2	
	Protein A	16			7	
	SpA CoI	64			85	
NC-passed, E^- (B+null)	Blank				<5	4·6
	Protein A				<5	5·8
	SpA CoI				175	145
NC-retained, E^- (B+Mϕ)	Blank				<5	<5
	Protein A				6	<5
	SpA CoI				125	194

* Cells/ml (1×10^6) of each cell population were incubated with or without stimulants for 24 hr and antiviral activity was determined by three different methods.
† Inhibition of the cytopathic effect.

the SpA CoI-stimulated supernatant was observed by the pH 2·0 treatment, while antiviral activity produced with the stimulation of Con A and Protein A, which was very low, became undetectable by this treatment.

Furthermore, antiviral activity was neutralized effectively by the incubation with sheep anti-α type IFN antibody (Table 4). These results indicate that the IFN produced in the SpA CoI-stimulation is consistent with the characteristics of α-type IFN.

Kinetics of augmentation of NK-cell activity and IFN production

Significant augmentation of NK-cell activity by SpA

Table 3. IFN titre after acid treatment of the culture supernatant

Supernatant Stimulant	Incubation time (hr)	IFN titre (i.u./10^6 cells/ml) Exp. 1 (no. 116) Non-treatment	Exp. 1 (no. 116) pH 2-treatment*	Exp. 2 (no. 117) Non-treatment	Exp. 2 (no. 117) pH 2-treatment*
Blank	24	<2	NT		
	48	<2			
Con A 10 μg/ml	24	4·5	<2		
	48	3·7	<2		
Protein A 10 μg/ml	24	4·1	<2		
	48	4·6	<2		
SpA CoI 0·005%	24	58	50	77	78
	48	52	57	124	102

* Supernatant was obtained after 24 or 48 hr of incubation of unfractionated cells with SpA CoI bacteria. The supernatant was adjusted to pH 2·0, left at 4° for 20 hr and readjusted to pH 7·2.

Table 4. IFN titre after neutralization by anti-IFN antibody

Supernatant (no. 084)	Dilution	IFN titre (i.u./10^6 cells/ml)* PBS control	Sheep anti-IFN antibody 1:1000	1:100
Medium only	1:1	<2	<2	<2
SpA CoI-stimulated	1:1	200	18	4
for 24 hr	1:10	25	<2	<2

* SpA CoI-stimulated supernatant was incubated with diluted anti-leucocyte IFNα at 37° for 2 hr. The anti-IFNα used in this experiment was capable of neutralizing 10 i.u. of IFNα at a dilution of 1:50,000.

CoI and IFNα stimulation appeared as early as 6–9 hr of incubation, reached maximal level up to 18 hr of incubation and declined gradually after 24 hr (Fig. 1). Augmentation by Protein A was observed, in contrast, with several hours delay and reached maximal level after 24 hr of incubation, where maximal level was always greater than those raised by SpA CoI and IFNα themselves. It should be noted that kinetics of augmentation of NK-cell activity by SpA CoI is quite similar to that by IFNα but not to that by Protein A.

The amount of IFN produced during each incubation time was determined (Fig. 1, upper panel). A significant amount of IFN was detected as early as 6 hr of incubation with SpA CoI and reached maximal level of 320 i.u. at 18 hr, continued during 48 hr culture. In contrast, no significant amount of IFN was detected after Protein A-stimulation (< 20 i.u. at 24 hr of incubation). These data suggest that SpA CoI-stimulated NK-cell augmentation is mediated through IFN produced in the culture but participation of IFN is much less probable in the Protein A-stimulation.

Potentiation of the NK-cell activity by the cultured supernatant

It was examined further whether the augmentation of NK-cell activity by the stimulants was mediated directly by the stimulants or by other soluble factors produced in the culture. As shown in Table 5, supernatants from Protein A- or SpA CoI-stimulated culture could augment the cytotoxicity of allogeneic and autologous (data not shown) lymphocytes against K-562 target cells effectively. Potentiating activity was found only in the culture supernatant from non-adherent cells and not from adherent cells, suggesting that the active factor(s) are derived from non-adherent lymphocytes.

Neutralization of IFN activity in the supernatant by anti-IFNα antibody

We investigated finally whether IFN produced in the

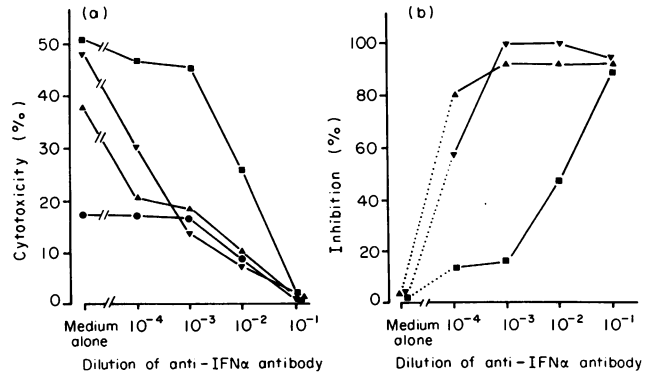

Figure 2. Effect of anti-IFNα antibody on the potentiation of NK-cell activity by the cultured supernatant. (a) Fresh lymphocytes were incubated for 24 hr with the supernatant which was obtained from Protein A-, SpA CoI-, or IFN-stimulated culture. Supernatant (300 μl) was treated with either equal volume of medium alone or anti-IFNα antibody for 2·5 hr at 37°, and then fresh lymphocytes ($6·6 \times 10^5$/60 μl) were mixed and incubated for further 24 hr. (b) Data of (a) was expressed as percentage inhibition:

$$\% \text{ Inhibition} = \frac{\% \text{ CTX by the stimulated sup. (anti-IFN}\alpha\text{-treated)} - \% \text{ CTX by the control sup. (anti-IFN}\alpha\text{-treated)}}{\% \text{ CTX by the stimulated sup. (medium alone)} - \% \text{ CTX by the control sup. (medium alone)}} \times 100$$

Table 5. Potentiating effect of the Protein A- or SpA CoI-stimulated supernatant on the cytotoxicity of peripheral blood lymphocytes*

Source of supernatant†			Cytotoxicity (%)	
Cell population	Stimulant	Effector cells	10/1§	5/1
Fresh medium		Unfractionated‡	16·1+3·6	11·6+2·6
Unfractionated	Blank	Unfractionated‡	16·2+2·8	11·2+2·1
	Protein A¶	Unfractionated‡	38·7+5·8	32·5+4·4
	SpA CoI**	Unfractionated‡	29·9+3·4	24·7+3·8
Non-adherent cells	Blank	Unfractionated‡	21·5+2·6	18·8+2·6
	Protein A	Unfractionated‡	42·4+6·4	30·6+5·2
	SpA CoI	Unfractionated‡	33·9+5·3	26·2+4·0
Adherent cells	Blank	Unfractionated‡	18·3+3·2	13·9+3·0
	Protein A	Unfractionated‡	19·6+2·7	15·7+2·7
	SpA CoI	Unfractionated‡	19·4+3·3	16·3+2·6

* Data represent mean of four experiments (numbers 138–141).

† Cells/ml (1×10^6) of each cell population were incubated with stimulant for 24 hr and the supernatant was collected. The supernatant was filtered through millipore membrane and added to the effector cell cultures at a final concentration of 50%.

‡ Allogeneic cells were used in these experiments. Similar results were obtained when autologous cells were employed.

§ E:T ratio.

¶ 10 μg/ml.

** 0·005%.

culture was actually responsible for the augmentation of NK-cell activity. The supernatant which was neutralized previously by goat anti-IFNα antibody was then added to the fresh lymphocyte cultures and its potentiating activity tested. As shown in Fig. 2a and b, potentiation by IFNα and SpA CoI was completely inhibited by the treatment with 1:1000 diluted anti-IFNα antibody. In contrast, no significant reduction was observed in the Protein A-stimulated supernatant. Significant enhancing activity remained after the removal of Protein A by IgG-conjugated Sepharose, followed by the neutralization of IFNα (data not shown). Employment of higher concentrations of anti-IFNα antibody caused non-specific suppression of NK-cell activity probably because of cellular cytotoxicity.

These data indicate clearly that IFN was a major effector molecule for the NK-cell augmentation in case of SpA CoI, while only a meager role was played by IFN for the Protein A-mediated NK-cell augmentation.

DISCUSSION

We have shown in this paper that both SpA CoI bacteria and its Protein A-potentiated human NK-cell activity strongly, and that the potentiation with SpA CoI could be due in most part to the IFN induced by the stimulation, while it was less probable that IFN might work in the potentiation with Protein A. It is well known that IFN and IFN inducers augment NK-cell activity in humans and mice. Of particular interest in NK-cell activation during these years has been the demonstration that NK-cell activity could be augmented by various bacterial (Wolfe *et al.*, 1977; Djeu *et al.*, 1979a) and chemical agents (Zarling *et al.*, 1979; Djeu, Heinbaugh, Holden & Herberman, 1979b), viruses and some tumour cells (Djeu *et al.*, 1980; Trinchieri *et al.*, 1978; Trinchieri & Santoli, 1978). These stimulants are known also as good inducers of IFN which seems, as a result, to act on NK cells or pre-NK cells to augment their killing activity.

Which type of IFN is more potent for the augmentation of NK-cell activity is a problem of great concern. It seems that both β-type of IFN which was derived from poly I:C-induced fibroblast cells (Zarling *et al.*, 1979), and type II (γ-type) IFN which was induced by tumour cells (Trinchieri *et al.*, 1978; Djeu *et al.*, 1980) were effective in the augmentation of NK-cell activity. Ley, Damme, Claeys, Weening,

Heine, Billian, Vermylen & Somer (1980) observed that the γ-type IFN was equally active compared with α- and β-type IFN in the NK-cell potentiation. Similarly, Rumpold, Kraft, Scheiner, Meindl & Bodo (1980) showed that both α- and β-type IFN were active to the same extent.

We found that SpA CoI bacteria induced α-type IFN in the culture. Some comment should be made, since it was reported that Staphylococcal enterotoxin A (SEA), which is a human T-cell mitogen, was a strong inducer of mitogen-type IFN (γ-type) (Johnson, Stanton & Baron, 1977; Langford, Stanton & Johnson, 1978). SpA CoI bacteria is, on the other hand, strong B-cell mitogens (Kasahara *et al.*, 1980) and so our result is not incompatible with the Johnson observation, if it is true that as in humans with α-type IFN is usually produced in the mouse by cells other than T cells when incubated with viruses and B-cell stimulants (Youngner & Salvin, 1973; Wietzerbin, Falcoff, Catinot & Falcoff, 1977).

Much less amount of IFN was induced with the Protein A-stimulation in our assay, although Protein A could augment NK-cell activity more strongly than SpA CoI bacteria. This indicates that Protein A-induced cytotoxicity is not mediated mainly by IFN but is rather due to direct activation of cytotoxic T cells or NK cells. In this regard, interesting observation was presented recently by Ratliff, McCool & Catalona (1981). They reported that Protein A induced significant amount of IFN production, which could augment NK activity in human peripheral blood lymphocytes. This seemingly conflicting result may be reconciled by the difference of IFN type, since they asserted that IFN produced by Protein A belonged to type II (IFNγ) because of its physicochemical nature and resistance to anti-IFNα antibody. Although Protein A induced only low titres of IFN in our system, far more IFN could be constantly produced by SpA CoI-stimulation (Ratliff *et al.*, 1981, did not test IFN production by SpA CoI), suggesting that IFN participates more directly in the SpA CoI-mediated NK-cell activation than in Protein A.

While Protein A was unable to activate the cytotoxic activity (against K-562 target) of purified T cells at least during the 1 day incubation period (Table 1), it is highly probable to activate T cells effectively in the presence of B cells or macrophages (e.g. unfractionated cells), since Protein A could activate T cells only in the presence of B cells or macrophages (Kasahara *et al.*, 1980). In fact, a considerable proportion of cytotoxic effector cells generated in the prolonged incubation with Protein A possessed T-cell-surface markers (data not shown). Similarly, Sumiya, Kano, Oshimi, Gonda & Takaku (1980) suggested that Protein A was capable of inducing strong cytotoxic T-cell activity against a B-lymphoid-cell line when incubated for 3 days. In our study T-cell-cytotoxic activity against various T- and B-cell lines was generated by the Protein A-stimulation during 1 day incubation (unpublished data). Alternatively, it is possible that Protein A induces some soluble factors other than IFN (such as T-cell-growth factor, IL-2), which successfully potentiate of NK cells, since it was recently suggested that IL-2 augments natural killer activity in mice (Henney, Kuribayashi, Kern & Gillis, 1981). In our preliminary observations, we found no significant production of IL-2 by SpA CoI-stimulation, suggesting that IL-2-like substance is of less importance in NK-cell activation by SpA CoI. In contrast, significant IL-2 activity was observed in the Protein A-stimulated culture (unpublished data). It may be highly probable that substances which act on T cells activate NK cells as well. Whether IL-2 works on NK cells in humans and affects their activity is of interest and the study is now in progress. These studies will be actually useful not only for the understanding of the mechanism of NK-cell activation *in vivo* and *in vitro* but for the immunopathological approaches in the various immunological disorders.

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