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Mechanism of stimulation of natural killer-cell cytotoxicity by interferon and an interferon-inducer in the rat

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Summary. The interferon-inducer Newcastle disease virus (NDV) was shown to augment cytotoxicity attributable to natural killer (NK) cells in all of the major lymphoid organs of W/Fu rats except the thymus. The levels of interferon isolated from the spleen following NDV inoculation correlated with the increase in splenic cytotoxicity from the same spleen. Spleen-derived interferon was shown to augment splenic cytotoxicity following intravenous inoculation, and to augment spleen cell cytotoxicity *in vitro*. Three major peaks of interferon type I were found in spleen homogenates corresponding to mol. wt of > 100,000, 29–33,000 and 19–23,000. All these fractions stimulated spleen cell cytotoxicity when tested *in vitro*. The rapid drop in splenic cytotoxicity 24 hr after NDV inoculation was associated with a rapid fall in interferon levels *in vivo*. The need for the continued presence of interferon for the stimulation of cytotoxicity was demonstrated when spleen cells pretreated with interferon for 4 hr *in vitro* lost their augmented cytotoxicity upon culturing for a further 20 hr in the absence of interferon. Although splenic cytotoxicity returned to control levels within 24 hr of a single $10^{7.3}$ EID_{50} dose of NDV, repeated doses of NDV maintained augmented cytotoxicity over a longer period. Spleen cells either taken from rats injected with NDV or pretreated *in vitro* with interferon showed a two-fold increase in the number of cytotoxic cells bound to W/FuG-1 target cells, with no change in the target binding-cell numbers. However, only the cells pretreated with interferon showed an increase in lytic efficiency.

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INTRODUCTION

Lymphoid cells showing naturally-occurring cytotoxicity towards a broad range of tumour and virus-infected cells *in vitro* have been described in rodents and man (Herberman & Holden, 1978; Santoli & Koprowski, 1979) A variety of agents has been shown to augment the cytotoxicity of these natural killer (NK) cells (reviewed by Welsh, 1978). Furthermore, most of the agents capable of augmenting NK cell cytotoxicity have been shown to induce interferon both *in vivo* (Djeu, Heinbaugh, Holden & Herberman, 1979a; Gidlund, Örn, Wigzell, Senik & Gresser, 1978) and *in vitro* (Trinchieri & Santoli, 1978; Einhorn, Blomgren & Strander, 1978; Shellam, Winterbourn & Dawkins 1980). Both type I (Djeu *et al.*, 1979a; Gidlund *et al.*, 1978; Shellam *et al.*, 1980) and type II (Djeu, Huang & Herberman, 1980) interferons have been shown to augment NK-cell activity, although the exact pathway(s) by which NK-cell activation occurs

has still to be elucidated. It has been shown that macrophages are an important source of interferon during NK-cell activation *in vivo* (Tracey, 1979; Djeu, Heinbaugh, Holden & Herberman, 1979b) and there is some evidence that NK cells themselves may produce interferon (Trinchieri, Santoli, Dee & Knowles, 1978; Minato, Reid, Cantor, Lengyel & Bloom, 1980). Recent evidence has shown interferon to be able to stimulate non-cytotoxic pre-NK cells to become lytically active (Saksela, Timonen & Cantell, 1979; Targan & Dorey, 1980) and to increase the lytic efficiency of the cytotoxic cells (Targan & Dorey, 1980). It has also been suggested that interferon may mediate some of its effects on NK cells via regulatory cells (Cudkowicz & Hochman, 1979).

In this study a rat model was used, as NK cells have been previously characterized in normal rats (Shellam, 1977) and in rats whose cytotoxicity was augmented by *Corynebacterium parvum* (Oehler, Lindsay, Nunn & Herberman, 1978; Flexman & Shellam, 1980) or tumour cells (Dawkins & Shellam, 1979a). Whilst the ability of interferon to augment mouse and human NK cells has been well studied, little is known about the effects of interferon on rat NK cells. Several features of rat NK cells make them a particularly relevant model of the human NK-cell system; in contrast to murine NK cells, human and rat NK cells are stable in short-term culture and their cytotoxicity does not decline with the age of the donor (Herberman & Holden, 1978). Therefore a study of the effects of interferon on rat NK cells is of particular interest. Recently, studies in this laboratory have shown that interferon produced in cultures of rat spleen cells and tumour cells augments NK-cell cytotoxicity (Shellam *et al.*, 1980). However, information on the kinetics of the effect of interferon on NK cells and on the mechanism of augmentation is lacking. Here we examine the variations in interferon levels *in vivo* during the process of augmentation of NK-cell cytotoxicity by Newcastle disease virus (NDV) and investigate the mechanism of augmentation by studying target–effector interactions.

MATERIALS AND METHODS

Animals

Inbred Wistar/Furth (W/Fu) rats were bred at the University of Western Australia. Age-matched males of 8–10 weeks were used, except where stated otherwise.

Tumours

The culture of the tumour cell lines W/FuG-1, P815, RBL-5, YAC-1 and K562 in RPMI 1640 with 10% foetal calf (FCS), hereafter referred to as medium, is described elsewhere (Dawkins & Shellam, 1979a).

Preparation of cell suspensions

Single-cell suspensions were prepared in medium. Thymus was finely minced and passed through a cotton wool plug. Spleen and lymph nodes were minced and passed through a fine nylon mesh before passage through cotton wool. Peritoneal exudate cells (PEC) were harvested by injecting 40 ml of medium which lacked FCS but contained 5 u./ml of heparin followed by two washes (450 *g*) through medium with an FCS underlayer. Peripheral blood leucocytes (PBL) were obtained as described elsewhere (Shellam & Hogg, 1977). Bone marrow cells were flushed from the femurs with medium. Thoracic duct lymph (TDL) was obtained by overnight drainage. A femoral vein cannula was used to infuse Dulbecco's A and B medium containing 1 u. of heparin/ml at a rate of 2 ml/hr during the collection of TDL. All cell preparation procedures were carried out at 4°.

Newcastle disease virus

A non-pathogenic strain of NDV, supplied by Dr V. Smith, Department of Agriculture, was propogated in the allantoic cavity of 11-day-old fertile chicken eggs and the resultant virus suspension was spun at 2000 *g* for 10 min and stored at −70°. Infectivity of the virus stock was determined by an egg infectious dose (EID_{50}) titration, 50% end point, as previously described (Fazekas de St. Groth & White, 1958).

Preparation of crude interferon

Thirty W/Fu rats, 4–6 weeks old, were injected with 0·5 ml intravenously (i.v.) of NDV ($10^{8.3}$ EID_{50} u./ml). Their spleens were removed 7 hr later, minced, homogenized in phosphate-buffered saline (PBS; (10% w/v) and centrifuged at 3000 *g* for 10 min. The supernatant was then dialysed in 0·1 M glycine HCl buffer pH 2·0 for 4 days at 4°, neutralized by dialysis against PBS, the volume adjusted to 30 ml with PBS, centrifuged at 100,000 *g* and sterilized by filtration. All steps were carried out at 4°. A control homogenate was prepared using the spleens of normal W/Fu rats. Serum from W/Fu rats injected with NDV was treated in the same manner as the spleen homogenate.

Animal inoculation
All injections were done by the i.v. route. In all experiments three rats/group were used and lymphoid cells pooled within a group. Uninjected rats were used as controls, unless otherwise stated.

Column chromatography
Crude interferon preparations were fractionated on a Sephadex G-100 column using PBS pH 7·3 as the column buffer. The following were used as molecular weight markers; bovine serum albumin 68,000, ovalbumin 43,000, pepsin 35,000, trypsin 23,300 and myoglobin 17,700.

Characterization of effector cells
Adult thymectomized, lethally-irradiated, marrow-reconstituted (ATX.BM) rats were prepared as described previously (Shellam, 1974). The plaque-forming cell responses of ATX.BM rats to sheep erythrocytes were reduced at least sixty-fold. The removal of adherent cells by adherence to plastic has been described previously (Flexman & Shellam, 1980).

^{51}Cr release assay
The 4-hr ^{51}Cr release assay (CRA) using microtitre trays (Falcon, Oxnard, Calif.) and 10^4 ^{51}Cr labelled target cells/well, and the calculation of percentage ^{51}Cr release, has been described in detail elsewhere (Dawkins & Shellam, 1979a). Effector to target ratios of 200:1, 100:1, 50:1, 25:1 and 12:1 were routinely used. Data are expressed in cytotoxic units (CU) which are defined as the slope of the linear regression curve which is the best fit to the points obtained by plotting percentage ^{51}Cr release against the number of effector cells, and is expressed as percentage ^{51}Cr release per 10^6 lymphoid effector cells. The correlation coefficient of the slope invariably exceeded 0·93. The spontaneous release was routinely 5%–10% of the total counts.

Assay for antibody-dependent cell-mediated cytotoxicity
The antibody-dependent cell-mediated cytotoxicity (ADCC) assay was performed as previously described (Flexman & Shellam, 1980). Briefly, 2×10^6 P815 cells were incubated for 1 hr at 37° in 0·4 ml of an appropriate dilution of hyperimmune rat anti-P815 antibody or 0·4 ml of medium in the presence of 200 μCi of $Na_2[^{51}Cr]O_4$ (Amersham), followed by three washes. The cells were then used as targets in the CRA.

Interferon assay
The assay for antiviral activity is described elsewhere (Shellam *et al.*, 1980). In the absence of an international reference standard for rat interferon, a laboratory standard which was prepared as described above, was used. This crude preparation protected rat embryo fibroblasts against infection with Semliki Forest virus (SFV) or Sindbis virus (SV), was ineffective against encephalomycarditis virus in mouse cells and its effect was abolished by treatment with trypsin as previously described (Shellam *et al.*, 1980). These properties in addition to the pH2 stability are consistent with the characteristics of a type I interferon. Thus the preparation is hereafter termed interferon. Antiviral units are expressed as the reciprocal of the highest dilution inhibiting 50% of the cytopathic effect of SFV on secondary rat embryo fibroblasts. Control homogenate had no antiviral activity.

Target binding assay
Spleen cells were depleted of erythrocytes by treatment with NH_4Cl. The number of leucocytes binding to the W/FuG-1 target cells was determined as follows: two million spleen cells and 2×10^6 tumour cells were mixed in a total of 0·2 ml in each well of a 96-well microtitre tray, centrifuged for 5 min at 250 ***g*** at room temperature and incubated for 40 min at 4°; the cells were then resuspended eight times with a pasteur pipette and the effector-target conjugates counted by phase microscopy. Tumour cells and leucocytes could be distinguished by size. At least 300 leucocytes were counted when determining the percentage of target binding cells.

Agar assay
The agar assay was a slight modification of a published technique (Targan & Dorey, 1980), the only difference being that the conjugates were formed as for the target binding assay and after being on ice for 40 min the conjugates were left at room temperature for 10 min before their resuspension in agar, to avoid clumping. The percentage of bound effector cells that were cytotoxic was calculated as follows: (percentage of dead targets in conjugates) − (fraction of spontaneous dead targets × the percentage of dead targets in conjugates). In addition, the frequency of cytotoxic leucocytes in the original population was calculated by: (percentage of bound cells that were cytotoxic at 4 hr) × (percentage of total leucocytes bound to W/FuG-1).

RESULTS

The effect of NDV on cytotoxicity in different lymphoid organs

The interferon-inducer NDV was shown to augment cytotoxicity two- to three-fold in the spleen and lymph nodes at 12 hr and 24 hr, respectively (Fig. 1). With the exception of the thymus the other lymphoid organs tested showed some increase in cytotoxicity within 24 hr, but to a lesser degree than the spleen or lymph nodes. Notably, augmented activity of PEC plateaued from 12 hr onwards. The only changes in the cell recoveries from the lymphoid organs were a 20%–30% transient lymphocytopenia at 12 and 24 hr in PBL and at 12 hr in the lymph nodes. This was followed by a two-fold increase in cell recoveries in lymph nodes over the 24–48 hr period and of PBL at 48 hr. The augmentation of splenic cytotoxicity was found to be dose dependent; at 12 hr a two- to three-fold increase was seen with doses in the range $10^{6\cdot3}$ to 10^{8} EID_{50} units as compared with a one and a half-fold increase with a 10^{5} EID_{50} u. dose (data not shown). The dose of NDV also affected the length of time required for augmented splenic cytotoxicity to return to normal levels; levels returned to normal after 36 hr using 10^{8} EID_{50} (Fig. 1) compared with 18 hr using $10^{7\cdot3}$ EID_{50} (Fig. 2).

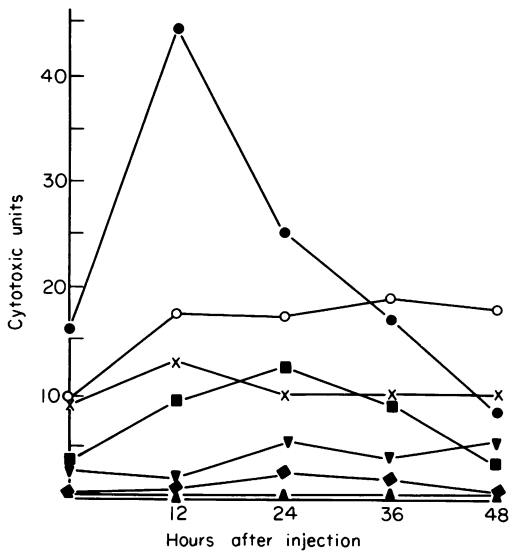

Figure 1. Kinetics of augmentation by NDV of cytotoxicity in lymphoid organs. Rats injected with NDV received a 10^{8} EID_{50} unit dose i.v. at the times indicated before assay. The cells were tested in a CRA against W/FuG-1 targets. (●) Spleen; (○) peritoneal exudate cells; (■) lymph node cells; (▼) thoracic duct lymphocytes; (▲) bone marrow; (△) thymus; (X) peripheral blood leucocytes.

Relationship between augmented cytotoxicity and interferon levels in the spleen and serum

Newcastle disease virus was injected into rats and the spleens were processed so that the interferon levels and cytotoxicity could be directly compared in the spleens of each group. Circulating interferon was measured in the serum. A three-fold augmentation in splenic cytotoxicity was seen over the 6–12 hr period with a return to control levels by 18 hr (Fig. 2). Splenic interferon levels peaked at $10^{3\cdot4}$–$10^{3\cdot7}$ u./ml during the 6–12 hr period, then dropped sixteen-fold between 12 and 18 hr. The serum interferon response was of shorter duration and peaked at 9 hr.

Ability of crude interferon to augment splenic NK cytotoxicity and ADCC

As a strong correlation was found between splenic cytotoxicity and spleen interferon levels following the administration of NDV (Fig. 2), the effect of i.v. inoculation of interferon produced in this way on splenic cytotoxicity was examined. An interferon-containing, spleen homogenate was found to augment cytotoxicity approximately two-fold between 6 and 12 hr when compared with uninjected controls, but even greater

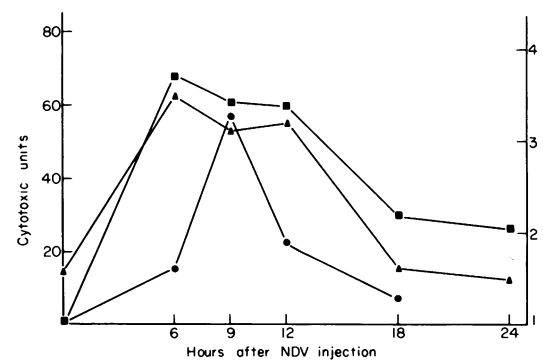

Figure 2. Relationship between augmented cytotoxicity and *in vivo* interferon levels. Rats injected with NDV were given $10^{7\cdot3}$ EID_{50} units i.v. at the times shown before assay. For each group (two rats) the spleens were minced and two-thirds, by weight, processed to extract the interferon (see Materials and Methods) and one-third was processed to give a single cell suspension. W/FuG-1 cells were used as targets in the CRA. (▲) Cytotoxicity; (■) spleen interferon titre; (●) serum interferon titre.

when compared with the effects of control homogenate (Fig. 3). The interferon-containing preparation also stimulated ADCC to antibody-coated P815 cells with a 2·4-fold rise at 12 hr when compared with control homogenate (Fig. 3).

Augmentation of NK cytotoxicity *in vitro* by interferon

The decline in splenic cytotoxicity after NDV inoculation coincided with the falling levels of interferon (Fig. 2). To examine whether this was a causative association, spleen cells were cultured *in vitro* for a total of 24 hr, with interferon being added at various times before assay. The increase in cytotoxicity was found to be time dependent ranging from one and a half-fold at 1 hr to three-fold at 24 hr (Table 1). In the same experiment cells which were cultured in the presence of interferon for 4 hr, and washed and recultured in the absence of interferon for 20 hr showed no increase in cytotoxicity above control levels. Control homogenate slightly stimulated cytotoxicity after pretreatment for 1–4 hr but was slightly suppressive if it was present for the full 24 hr.

Mechanisms of augmentation of cytotoxicity by NDV and interferon

To gain an insight into the mechanisms responsible for

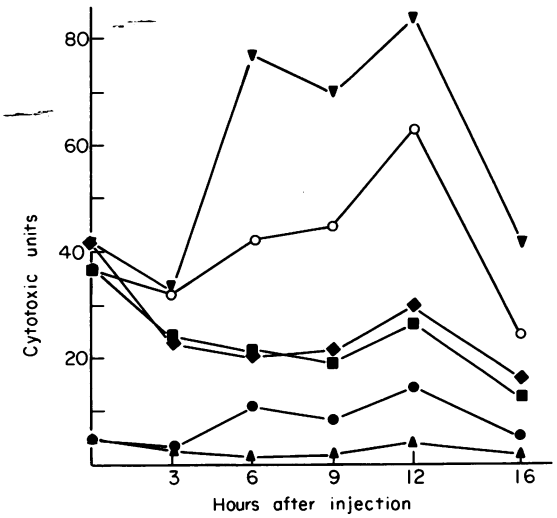

Figure 3. Effect of interferon on NK cytotoxicity and ADCC in the spleen. Interferon-containing homogenate, 0·5 ml at 640 u./ml, was injected i.v. into rats (3 per group) and the spleens taken at the times shown. Control homogenate, 0·5 ml, was injected into rats as a control. Spleen cells within each group were pooled and tested in a CRA against W/FuG-1, P815 and antibody coated P815 target cells. (▾) Interferon, W/FuG-1; (●) interferon, P815; (○) interferon, antibody coated P815; (▲) control homogenate, W/FuG-1; (△) control homogenate, P815; (■) control homogenate, antibody-coated P815.

Table 1. Augmentation of cytotoxicity by interferon *in vitro* and the loss of augmented cytotoxicity on continued culture in the absence of interferon*

Treatment	Culture time before CH or INF added† (hr)	Culture time in presence of CH or INF (hr)	Culture time after CH or INF removed (hr)	Cytotoxic units‡ ± SE
Medium	—	—	—	22·6 ± 1·5
CH	23	1	0	30·0 ± 1·3
INF	23	1	0	44·9 ± 1·7
CH	20	4	0	32·2 ± 0·8
INF	20	4	0	65·2 ± 4·2
CH	11	13	0	23·4 ± 0·4
INF	11	13	0	50·8 ± 3·0
CH	0	24	0	14·4 ± 0·4
INF	0	24	0	42·4 ± 3·2
Medium	0	4	20	16·4 ± 0·7
CH	0	4	20	17·8 ± 1·8
INF	0	4	20	18·3 ± 0·5

* Spleen cells were treated with NH_4Cl and then cultured with or without interferon for the times shown. Cells (10 ml at 10^7/ml) were incubated with interferon at $1{\cdot}3 \times 10^2$ u./ml and control cells were incubated in an equivalent dilution of control homogenate. After the incubation period with interferon or control homogenate, cells were washed twice in medium, after which they were either assayed directly or returned to culture for 20 hr before assay.

† CH, control homogenate; INF, interferon.

‡ Cytoxicity against W/FuG-1 target cells.

Table 2. The effect of NDV injection or *in vitro* interferon pretreatment on target binding cell numbers and frequency of cytotoxic cells in W/Fu rats

Experiment*	Group	Target-binding cells (%)†	Cytotoxic leucocytes (%)‡ 1 hr	4 hr	Frequency of cytotoxic cells (%)§	Cytotoxic units (±SE)¶
I	Control	13·0	8	21	2·73	—
	INF 2 hr	12·0	51	54	6·48	—
II	Control	13·0	7	20	2·60	—
	INF 2 hr	13·0	48	51	6·63	—
III	Control	13·3	—	15	2·00	73·6±4·5
	INF 2 hr	12·2	—	34	4·15	111·6±8·4
	INF 24 hr	15·5	—	26	4·03	135·3±9·1
IV	Control	14·0	11	32	4·48	15·8±1·2
	NDV 12 hr	15·0	19	60	9·00	25·9±3·3
V	Control	11·0	11	20	2·20	—
	NDV 4 hr	11·5	28	43	4·95	—
	NDV 24 hr	10·0	14	22	2·20	—

* Experiments I, II and III: spleen cells treated with NH_4Cl were incubated for the times shown at 10^7 cells/ml in the presence of interferon at $1{\cdot}3 \times 10^2$ u./ml, washed twice and assayed. Experiments IV and V: NDV was injected i.v. at the appropriate time before assay, $10^{7{\cdot}3}$ EID_{50} units in 0·5 ml.

† Percentage of cells binding to W/FuG-1 targets.

‡ Percentage of cytotoxic cells against W/FuG-1 in the agar assay.

§ Percentage target binders × percentage cytotoxic leucocytes, appropriate background controls subtracted (see Materials and Methods).

¶ Cytotoxicity against W/FuG-1 in the CRA.

the observed increase in NK-cell cytotoxicity following interferon or NDV stimulation, an assay which allows the frequency of cytotoxic cells in a population to be determined by counting dead tumour cells conjugated to viable effector cells was used. As shown in Table 2, spleen cells pretreated with interferon *in vitro* showed a 2·4- to a 2·6-fold increase in the frequency of cytotoxic cells with no increase in the number of target binding cells (experiments I and II). In addition it was shown that *in vitro* interferon pretreatment enhanced the lytic efficiency of the cells as the maximum percentage of cytotoxic effector cells was seen after 1 hr as compared with 4 hr for control cells (experiment I and II). It should be noted that the percentage of cytotoxic cells in the agar always plateaus out between 3 and 8 hr, after which the number of conjugates/plate rapidly declines due to disintegration of conjugates (data not shown). In experiment III it was found that the two-fold increase in the frequency of cytotoxic cells was not further increased by an extended pretreatment with interferon *in vitro* for 24 hr. The *in vivo* administration of NDV also resulted in a two-fold increase in the frequency of cytotoxic cells. However, no increase was seen in the cytotoxic efficiency of the cells in the NDV group as the percentage of bound cytotoxic cells in the 1-hr agar assay was similar for both control (11/32, 34·4%) and NDV (19/60, 31·7%) groups, taking the 4-hr agar assay result as being the maximum level of cytotoxic cells for each group (experiment IV). Finally, it was shown that after NDV administration the frequency of cytotoxic cells returned to control levels after 24 hr, following a two-fold increase in the frequency of cytotoxic cells at 4 hr (experiment V).

The effect of repeated doses of NDV on splenic cytotoxicity

Since splenic cytotoxicity after NDV injection returns to normal by 24 hr (Fig. 2), repeated doses of NDV were given to determine if augmented cytotoxicity could be maintained over a longer period of time. Whilst a single dose of NDV augmented cytotoxicity at 10 hr only, multiple doses at 10 and 46 hr or 10, 46 and 82 hr restimulated cytotoxicity two-fold (Table 3).

Table 3. Effect of repeated doses of NDV on splenic cytotoxicity*

Time of NDV injection before assay (hr)	Cytotoxic units ± SE
Control	18·5 ± 0·8
10	37·6 ± 2·0
36	14·4 ± 1·0
46	15·5 ± 0·4
72	16·0 ± 0·8
82	15·0 ± 0·9
10, 46	43·0 ± 1·2
10, 46, 82	37·9 ± 2·9

* Rats were injected with $10^{7.3}$ EID_{50} units of NDV i.v. in 0·5 ml. Spleens were taken at the times shown after injection. In the case of multiple injections spleens were taken 10 hr after the final injection. The spleen cells were tested against W/FuG-1 targets in a CRA.

Molecular weight analysis of type I interferons found in the spleen following NDV administration

The interferon-containing spleen homogenate (3 ml at 2560 u./ml) was fractioned on a Sephadex G-100 column. Twelve 24-ml fractions were collected and each fraction concentrated eight-fold to give 3 ml/fraction. The twelve fractions were tested for their ability to augment NK-cell cytotoxicity *in vitro* and for their interferon titre, the results of which were plotted against the average molecular weight of each fraction (Fig. 4). There were three major peaks of anti-viral activity in the fractions, corresponding to the mol. wt ranges > 100,000, 29–33,000 and 19–23,000. The fractions with peak interferon titres were also the most effective at augmenting NK-cell cytotoxicity *in vitro* (Fig. 4). The results shown in Fig. 4 were reproduced in a second experiment and the reduced interferon titre and *in vitro* stimulation of spleen cell cytotoxicity observed here with the 28,000 Dalton fraction, was found to occur in fractions of 24,000–28,000 Daltons (data not shown). Furthermore, when control homogenate was fractionated and the fractions tested for their effect on NK-cell cytotoxicity *in vitro*, the levels of cytotoxicity did not vary beyond ±5% of the medium control value.

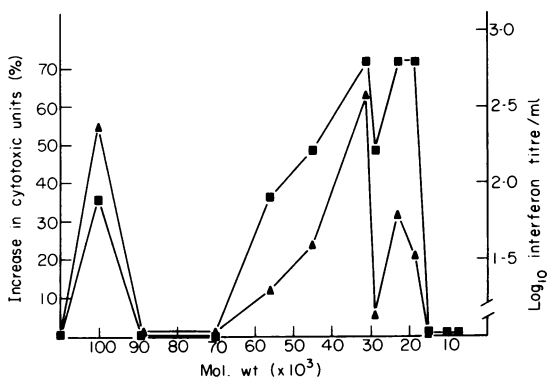

Figure 4. Sephadex G-100 fractionation of an interferon-containing spleen homogenate. Three millilitres of interferon-containing spleen homogenate 2560 u./ml was fractionated on a Sephadex G-100 column. The details of the collected fractions and molecular weight markers used are given in the text. Anti-viral activity and the capacity to augment spleen cell cytotoxicity *in vitro* were measured for each fraction. Cytotoxicity was tested by adding 0·1 ml of the fraction directly into the wells of the microtitre tray during the CRA, the total volume in each well being kept at 0·2 ml; (▲) cytotoxicity; (■) interferon titre.

Characterization of the effector cells after augmentation of cytotoxicity by NDV

The effector cells mediating cytotoxicity after NDV injection were shown to closely resemble the NK cells of normal rats in their properties (Table 4). The effector cells were shown to be non-adherent, non-T cells with the cytolytic specificity of NK cells (Table 4). The cells mediating ADCC in the control and NDV-augmented groups were also found to be non-adherent and non-T cells (Table 4). In the adherence experiment 10% of the cells were recovered as adherent, 80% as non-adherent and 10% were lost in handling.

DISCUSSION

It was shown that the intravenous administration of the interferon-inducer NDV increased cytotoxicity in all of the major lymphoid organs except the thymus. Therefore, although some migration during the process of augmenting cytotoxicity of the lymphoid organs cannot be ruled out, the concomitant increase in cytotoxicity of the major lymphoid organs suggest that NK-cell activation by NDV is largely due to effects *in situ* rather than migration. The effector cells augmented by NDV *in vivo* were shown to closely resemble the NK cells of normal rats in their characteristics, being non-adherent, non-T cells with the cytolytic specificity of NK cells. These characteristics have also been shown for other NK-cell inducers in the rat which include *C. parvum* (Oehler, *et al.*, 1978;

Table 4. Characteristics of splenic effector cells following NDV-induced augmentation of cytotoxicity

Experiment	Group*	Cytotoxic units W/FuG-1	P815	ā P815¶	RBL-5	YAC-1	K562
I	Control	40·6	7·2	—	5·4	42·4	17·7
	NDV	76·9	12·0	—	9·2	96·7	29·0
II	Control UNF†	30·2	9·7	30·6	—	—	—
	Control NA	25·3	8·1	33·7	—	—	—
	Control ADH	3·0	0·7	3·3	—	—	—
	NDV UNF	74·7	24·4	67·8	—	—	—
	NDV NA	53·3	18·5	57·0	—	—	—
	NDV ADH	6·6	0·6	7·3	—	—	—
III	Control‡	14·8	1·3	13·9	—	—	—
	NDV	31·2	2·1	29·4	—	—	—
	Control ATX-BM§	5·1	0·2	17·0	—	—	—
	NDV ATX-BM	18·7	0·8	60·1	—	—	—

* Rats injected with NDV received 0·5 ml of EID_{50} $10^{8·3}$ u/ml i.v. 12 hr before assay.

† Spleen cells separated according to adherence properties to plastic. NA, non adherent; ADH, adherent.

‡ Sham thymectomized at 6 weeks of age, but were not irradiated or reconstituted with bone marrow.

§ Thymectomized at 6 weeks of age. *X*-irradiated at 1000/rad and reconstituted with bone marrow cells from ATX-BM donors at 10 weeks of age and assayed at 17 weeks of age.

¶ ā P815, antibody-coated P815.

Flexman & Shellam, 1980) and tumour cells (Dawkins & Shellam, 1979b). Newcastle disease virus has been shown to augment NK-cell cytotoxicity 24 hr after injection in mice (Gidlund *et al.*, 1978). However, the experiments in this study have shown that augmentation can be detected as early as 4 hr after injection with a plateau of heightened activity between 6 and 12 hr.

A strong correlation was seen between interferon levels and cytotoxicity in the same spleen following NDV challenge. Interferon levels were shown to increase and then decline in conjunction with NK-cell cytotoxicity after inoculation of the virus. The kinetics of interferon production in the spleen and serum were found to be similar to that observed previously with NDV in the rat (Billiau & Buckler, 1970), with spleen levels always greater than that of the serum. The crude interferon-containing spleen homogenate derived from the spleens of rats injected with NDV was shown to augment splenic NK-cell cytotoxicity and ADCC, the kinetics of the response being similar to those observed for NDV. The concomitant augmentation of ADCC supports the concept that the cells mediating natural cytotoxicity and antibody-dependent cytotoxicity are closely related (Ojo & Wigzell, 1978). A similar correlation between augmented NK-cell cytotoxicity and ADCC was seen after administration of *C. parvum* (Flexman & Shellam, 1980).

The advantage of the approach taken in this study is that the crude interferon preparation used was extracted from the spleen following NDV challenge and therefore contained the different molecular weight forms of interferon type I produced *in vivo*. This point is particularly relevant in view of the heterogeneity in the cellular origin and molecular weight forms of interferon induced by NDV *in vivo* (Wagner & Smith, 1968; Bocci, Russi-Sorce, Cirri, Rita & Cantagalli, 1968) and because little is known about the regulation of NK cells by such different interferons. Hence, studies are required with a crude interferon preparation before the various components are analysed. Furthermore, although it might be argued that certain intracellular factors not normally released *in vivo* may have been present in the interferon-containing spleen homogenate, a control homogenate was also used so that the effects observed can be directly attributed to NDV. Fractionation of the crude interferon homogenate on Sephadex G-100 revealed peaks of antiviral activity at > 100,000, 29–33,000 and 19–23,000 Daltons, and these fractions were able to augment the cytotoxicity

of spleen cells *in vitro*. Thus it appears that all of the different molecular weight forms of type I interferon induced by NDV can augment NK-cell activity.

It was shown that spleen cells incubated for periods between 1 and 24 hr with interferon *in vitro* showed augmented cytotoxicity. In contrast, cells pretreated with interferon *in vitro* for 4 hr, washed and recultured for 20 hr without interferon lost their augmented cytotoxicity. This observation supports the *in vivo* data in which the decline in NK-cell cytotoxicity was accompanied by a reduction in interferon titre. The continued presence of interferon may be required for cytotoxicity to remain at an augmented level, particularly as it has been shown that the degree of augmentation of NK cell cytotoxicity *in vitro* is proportional to the concentration and time of exposure to interferon (Senik, Kolb, Örn & Gidlund, 1980; Silva, Bonavida & Targan, 1980). Although the cytotoxicity of NK cells declines in the absence of interferon, the ability to respond to further contact with interferon has been demonstrated in the human system. Thus additional exposure to interferon stimulated cytotoxicity to return to the previous augmented levels, declining again in the absence of further contact (Silva *et al.*, 1980) and this cyclic response was shown to be a property of pre-NK cells. The results of the present study suggest that the same process operates *in vivo*; cytotoxicity which was stimulated two to three-fold by NDV returned to control levels after 24 hr but was restimulated by repeated injections of NDV.

Examination of target–effector cell interactions revealed that NDV injection does not change the total number of spleen cells capable of binding to W/FuG-1 target cells, but does increase two-fold the percentage of cells bound that are cytotoxic to the targets. Interferon pretreatment of spleen cells *in vitro* produced the same effects as NDV but in addition increased the lytic efficiency of the effector cells. Interferon-pretreated human effector cells also have an increased lytic efficiency (Targan & Dorey, 1980). Although NDV injected *in vivo* increased the number of bound cytotoxically active cells, there was no increase in their lytic efficiency. Whether this latter observation is due to *in vivo* regulatory effects or an insufficient localized concentration of interferon is as yet unknown. However, as interferon concentration has been shown to be important *in vitro*, in that NK-cell cytotoxicity increases with increasing interferon concentration (Senik *et al.*, 1980, Silva *et al.*, 1980), the levels of interferon produced *in vivo* by injecting NDV might not have been sufficient to increase the lytic efficiency of the cells. Further studies are in progress to determine the effect of interferon on mature and pre-NK cells, in particular the levels required both to stimulate pre-NK cells into lytic activity, and to increase the lytic efficiency of both mature and pre-NK cells.

Finally, whilst the observations made in this study have been attributed to interferon type I on the basis of indirect evidence, it should be noted that purified preparations of interferon have been used in studies in man (Trinchieri & Santoli, 1978; Targan & Dorey, 1980) and mice (Gidlund *et al.*, 1978; Senik *et al.*, 1980). In addition, antibodies to interferon have been used in the mouse model (Gidlund *et al.*, 1978; Senik *et al.*, 1980). Hence, the direct demonstration of the role of interferon type I in stimulating NK cells in rats will only be possible when these reagents become available.

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