

Inhibition of Natural Killer Cell Activity by Antigen-Antibody Complexes

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The natural killer (NK) cell activity of human peripheral blood mononuclear cells was found to be inhibited by precipitated tetanus toxoid anti-tetanus toxoid complexes (Te/aTe) as well as soluble Te/aTe. Preincubation of the immune complexes with protein A decreased the inhibition of NK cell activity. When mononuclear cells were preincubated with interferon (IF) or interleukin 2 (Il-2) before incubation with Te/aTe, the immune complex-induced inhibition was decreased, while IF or Il-2 added after incubation with the immune complexes had no effect. Using NK cell-enriched suspensions in a single cell agarose assay, the immune complexes were shown to inhibit NK cell activity by inhibiting the formation of effector/target cell conjugates.

Key words: Fc-receptor; immune complexes; natural killer cells.

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Natural killer (NK) and killer (K) cells are found within the same subpopulation of lymphocytes, morphologically identified as large granular lymphocytes (LGL), that comprise about 5% of peripheral blood lymphocytes. LGL have been characterized as non-phagocytic, non-adherent cells that lack surface receptors for the C3b fragment of the third component of complement but contain low affinity receptors for sheep erythrocytes. The majority of NK cells have Fc receptors with low reactivity with human monomeric IgG, while K cells can be separated into subpopulations with high or low reactivity (7). The same cells (NK/K cells) seem able to mediate both NK cell activity and antibody dependent cellular cytotoxicity (ADCC) (19). The Fc receptors allow K cells to bind antibody-coated target cells and mediate ADCC, whereas the importance of the Fc receptor in mediating NK cell cytotoxicity still has to be clarified. Extensive studies have, however, shown that IgG antibodies do not play a major role in NK cytotoxicity *in vitro* (15), but NK cell activity is inhibited by certain kinds of antigen/anti-

body complexes and aggregates of IgG (14, 23).

The aim of this work has been to further characterize the mechanism by which NK cells are inhibited by immune complexes.

MATERIAL AND METHODS

Effector cells

Mononuclear cells were isolated from heparinized blood (125 IU/ml blood) by Ficoll Isopaque Gradient centrifugation (2). The cells were washed three times with Hanks' buffered salt solution (HBSS) and resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS) to a concentration of 2×10^6 cells/ml. In some experiments 1.5 ml of 4×10^6 cells/ml were incubated at 37°C on 60 mm plastic Petri dishes (Teknunc A/S Roskilde, Denmark) for 1 h. The non-adherent cells were aspirated, centrifuged (1000 g, 10 min) and resuspended in RPMI with 10% FCS to a final concentration of 2×10^6 cells/ml. This

suspension contained 0–2% peroxidase positive cells (26). In other experiments NK cell-enriched suspensions were prepared on discontinuous Percoll (Pharmacia Chemicals, Uppsala, Sweden) gradients as described previously by Timonen et al. (24).

Target cells

The human leukemia cell line K 562 was maintained in continuous culture in RPMI 1640 supplemented with 10% heat inactivated FCS. To $2-8 \times 10^5$ target cells in 1 ml were added 0.1 mCi of $\text{Na}_2^{51}\text{CrO}_4$ (New England Nuclear, Boston, Mass., U.S.A.) specific activity 350–600 mCi/mg. The cells were incubated with the isotope for 1 h at 37°C. The labelled cells were washed through a 4 ml cushion of heat-inactivated FCS. The cell pellet was resuspended in RPMI with 10% heat-inactivated FCS to a final concentration of 5×10^4 cells/ml.

Cytotoxic assay

NK cell activity was measured in triplicate (SD = 0.1–3.0%) at effector:target cell ratio 40:1. 400 µl of the labelled target cell suspension was mixed with 400 µl of effector cells and 200 µl of RPMI + 10% heat-inactivated FCS. After incubation for 4 h at 37°C the tubes were centrifuged (1000 g, 10 min), 500 µl of each supernatant was transferred to new tubes, and the radioactivity was counted in a Packard scintillation gamma counter. The experimental release (R) was calculated by the formula:

$$R = \frac{\text{activity released in the supernatant}}{\text{total activity}} \times 100\%$$

The specific release was the difference between experimental release in the presence of effector cells and experimental release when target cells were incubated with medium only. The within-assay determinations gave a coefficient of variation of 2.9%. As the percent specific release employing cells from different donors varied between 16.7% and 54.0%, the experimental data are given in percentages of the controls

(NK cell activity measured without the presence of immune complexes) (mean $\pm$ SD) for the sake of statistical evaluations. The day-to-day determinations on samples from the same donors gave overall coefficients of variation of 20%.

Single cell agarose assay

Non-adherent mononuclear cells or NK cell-enriched suspensions and unlabelled K 562 target cells were used as effector and target cells, respectively.

The assay was performed as described by Grimm et al. (11) with minor modifications. A 0.5% agarose solution in RPMI was kept in a waterbath at 50°C. 2.5×10^5 effector cells and 2.5×10^5 target cells were mixed in a total volume of 0.25 ml of RPMI with 10% FCS. The cells were centrifuged at 500 g for 5 min and incubated for 5 min at 37°C. During this time some of the lymphocytes were bound to target cells and formed effector/target cell conjugates. Subsequently, the pellet was resuspended by shaking for 3 s on a whirlmixer. The cells were then added to 0.6 ml of the agarose solution, which had been precooled to about 40°C, resuspended with a Pasteur pipet and added dropwise from a height of 30 cm onto 90 mm plastic Petri dishes (Teknunc A/S, Roskilde, Denmark). A thin layer of agarose which contained the cells was formed at the bottom of the dishes. The percentage of conjugates, referred to as the percentage of target binding cells (% TBC), was determined by counting the number of lymphocytes bound to target cells and the total number of lymphocytes. At least 400 lymphocytes were counted.

Interferon treatment of effector cells

0.1 ml of 10^5 IU/ml of α -interferon (IF) was added to 0.9 ml of 2×10^6 effector cells/ml. IF was kindly provided by Dr. Robert Jørdal, The Blood Bank, Copenhagen County Hospital, Gentofte, Denmark. After 1 h of incubation at 37°C the cells were washed three times with RPMI + 10% FCS and resuspended in the same medium in the appropriate effector cell concentration.

Interleukin 2 (IL 2) treatment of effector cells

Purified IL-2 was purchased from Boehringer-Mannheim, Mannheim, W. Germany, and preincubated with 2×10^6 effector cells/ml for 1 h at 37°C at a final concentration of 10%. IL-2 was present during the incubation with target cells as well.

Tetanus toxoid anti-tetanus toxoid complexes

The purified preparation XXVII (3 mg/ml) of tetanus toxoid from Statens Seruminstitut (Copenhagen, Denmark) and tetanus immunoglobulin human (aTe) tetabulin from Immuno, Wien, Austria (250 units/ml) were used diluted in veronal buffered saline (VBS; 1.02 g sodium barbital and 8.22 g NaCl in 1 l of distilled water, pH 7.4).

Immune complexes were formed in accordance with the principles used for preparation of a provisory reference preparation for detection of immune complexes by an I.U.I.S. standardization subcommittee (Dr. U. Nydegger, SRK, Wankdorfstrasse, 10, 3000 Bern 22, Switzerland). A quantitative precipitation test was carried out, and immune complexes were prepared in VBS at different antigen/antibody ratios. The Te/aTe mixtures were incubated at 37°C for 1 h and separated into insoluble (= precipitated) and soluble fractions by 30 min centrifugation at 1500 g (22°C). The concentrations of the Te/aTe preparations are given in terms of µg tetanus toxoid per ml. The controls (Te and aTe) were used at concentrations corresponding to the content of the Te/aTe preparations.

Precipitated Te/aTe (100 µg Te/ml) was prepared at two times antibody excess. The pellet and 20% of the original volume left in the tubes were whirlmixed for 30 s, and the content dispersed by passage through a tuberculin needle 3 times. Soluble Te/aTe (Te/aTe "S") (160 µg Te/ml) was prepared at four times antigen excess; 80% of the supernatant was collected. The samples were stored in small aliquots at -80°C.

Precipitated Te/aTe diluted 1:10 in VBS corresponded to strongly immune complexed posi-

tive sera by quantitation in the complement consumption step of the PEG-CC assay (3), while Te/aTe diluted 1:100 were negative in the test. As expected these soluble immune complexes (Te/aTe "S") were not detectable by analysis in the complement consumption step of the PEG-CC assay (3, 13).

Te/aTe incubation with effector cells

2×10^6 effector cells/ml was mixed with Te/aTe diluted 10-fold and preincubated for 1 h, 37°C. In some experiments incubation of Te/aTe was followed by incubation with IF or IL-2. In other experiments the effector cells were preincubated with IF or IL-2 followed by addition of immune complexes. In most experiments the cells were washed before incubation with target cells, but in one series of experiments the cells were washed or unwashed before incubation with target cells.

Protein A treatment of Te/aTe

The Te/aTe immune complexes were incubated with free, soluble protein A prepared from *Staphylococcus aureus* (Pharmacia) 0.25 mg/ml for 2 h at 37°C, washed twice in RPMI with 10% FCS and incubated with effector cells for 1 h. The controls (Te/aTe not incubated with protein A) were washed in parallel.

RESULTS

Effects of Te/aTe on NK cell activity

When mononuclear cells from healthy donors were incubated with various concentrations of the immune complexes Te/aTe, the NK cell activity was inhibited in a dose-dependent manner (Fig. 1). Incubation of mononuclear cells with either the antigen Te or the antibody aTe did not inhibit the NK cell activity (Fig. 1).

Preincubation of immune complexes with protein A (Fig. 2) before incubation with effector cells reduced the inhibition of NK cell activity by Te/aTe. Protein A treatment of Te/aTe increased the NK activity from 4.9% to

NK ACTIVITY

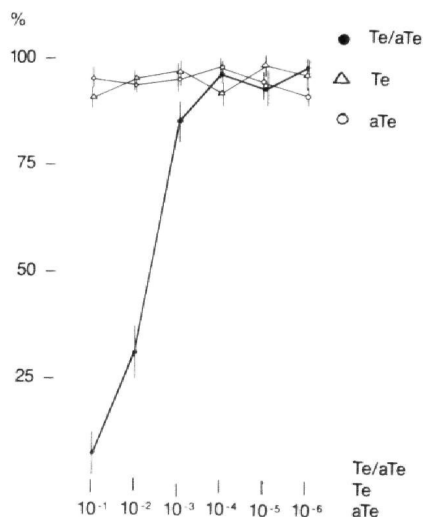


Fig. 1. Inhibition of NK cell activity by Te/aTe immune complexes: mononuclear cells were incubated with precipitating immune complexes Te/aTe, Te or aTe and washed. NK cell activity was measured against K 562 target cells. The results are given in percentages of the controls (mean $\pm$ SD of eight experiments).

24.8% at Te/aTe diluted 1:10, from 24.9% to 77.1% at Te/aTe diluted 1:100, and from 64.8% to 105.0% at Te/aTe diluted 1:1000. Protein A by itself had no effect on NK cell activity.

The inhibition of the NK cell activity was irreversible as washing of the effector cells after incubation with Te/aTe did not alter the level of inhibition (Table 1). The soluble immune

NK ACTIVITY

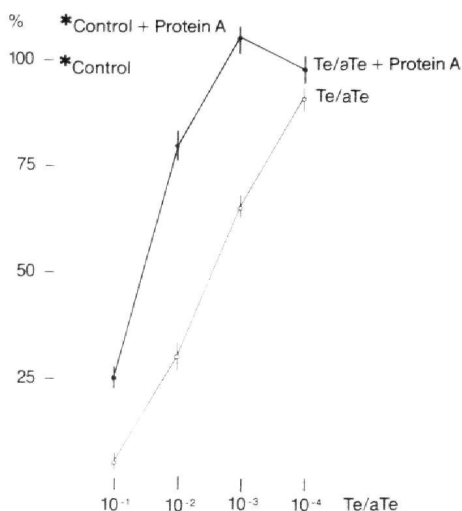


Fig. 2. Protein A-induced reduction in effect of Te/aTe: the immune complexes Te/aTe were incubated with protein A before incubation with mononuclear cells. The results are given in percentages of the controls (mean $\pm$ SD of three experiments).

complexes (Te/aTe "S") prepared in antigen excess gave identical results (Table 2).

When NK cell activity was almost totally suppressed by Te/aTe diluted 1:10, addition of IF or Il-2 did not boost the NK cells. Preincubation of the NK cells with IF or Il-2 followed by addition of Te/aTe gave similar inhibition of NK cell activity but at another level. (Table 3).

The results shown were obtained by the use of

Table 1
Effect of washing the mononuclear cells after incubation with Te/aTe

Concentration of Te/aTe	NK activity (%)						Control
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	
Washed	1.3 $\pm$ 1.4	20.0 $\pm$ 3.7	86.7 $\pm$ 4.4	85.3 $\pm$ 7.8	86.7 $\pm$ 6.7	98.2 $\pm$ 6.4	100
Unwashed	0.4 $\pm$ 0.3	22.2 $\pm$ 5.7	84.4 $\pm$ 3.6	94.2 $\pm$ 2.9	83.1 $\pm$ 4.3	96.9 $\pm$ 3.8	100

Mononuclear cells were incubated with various concentrations of the immune complexes Te/aTe and used washed or unwashed at testing for NK cell activity. Results are given in percentages of the controls (mean $\pm$ SD of three experiments). The specific release by cells from the different donors varied between 16.7 and 52.2%.

Table 2
Effect of Te/aTe and Te/aTe "S" on NK cell activity

Concentration	NK activity (%)				Control
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	
Te/aTe	6.1 ± 4.8	62.4 ± 3.8	112.7 ± 7.2	90.1 ± 4.1	100
Te/aTe "S"	15.3 ± 3.2	65.7 ± 7.1	101.1 ± 3.5	104.9 ± 2.6	100

Mononuclear cells were incubated with various concentrations of the precipitated immune complexes Te/aTe and the soluble immune complexes Te/aTe "S", washed and tested for NK cell activity. The results are given in percentages of the controls (mean ± SD of three experiments). The specific release by cells from different donors varied between 18.1 and 23.4%.

mononuclear cells, separated on Ficoll Isopaque, but experiments with mononuclear cells depleted for plastic-adherent cells were made in parallel, and gave identical results (data not shown).

Effects of Te/aTe on the recognition and adhesion step

To study whether the incubation of effector cells with immune complexes inhibited cytotoxicity by interference with the recognition and adhesion step or by blocking the final killing event, the adhesion of monocyte-depleted cells to target cells after incubation with Te/aTe and Te/aTe "S" was studied in a single cell agarose assay (Table 4). The data clearly show that both Te/aTe and Te/aTe "S" immune complexes inhibit the adhesion step. Suspensions enriched in NK cells by Percoll gradient separation formed

a greater number of effector/target conjugates than unfractionated mononuclear cells.

DISCUSSION

This study demonstrates that the precipitated antigen-antibody complexes Te/aTe inhibit NK cell activity at concentrations judged positive in a conventional assay for circulating immune complexes. Similar results were obtained by soluble immune complexes. The antigen or antibody alone does not have any inhibitory effect. This is in agreement with previous findings by others, which show that a number of *in vitro* prepared antigen-antibody complexes or aggregates of IgG, as well as serum containing immune complexes, inhibit NK cell activity (6, 14, 23).

In the ADCC system cytotoxicity is significantly inhibited when protein A is added, presumably due to binding of protein A to avail-

Table 3

Effect of IF and Il-2 on Te/aTe-induced inhibition of NK cell activity

Incubation 1	NK activity (%)	
	Incubation 2	NK activity %
-	-	100
-	IF	147.3 ± 7.4
-	Il-2	140.4 ± 5.2
Te/aTe	-	4.1 ± 2.7
Te/aTe	IF	4.5 ± 3.2
Te/aTe	Il-2	4.6 ± 2.8
IF	Te/aTe	53.4 ± 8.6
Il-2	Te/aTe	64.9 ± 7.0

Mononuclear cells were incubated with or without Te/aTe diluted 1:10 followed by stimulation with IF or Il-2, or preincubated with IF or Il-2 followed by incubation with Te/aTe. Results are given in percentages of controls (mean ± SD of three experiments). The specific release by cells from different donors varied between 17.0 and 54.0%.

Table 4

Effect of Te/aTe and Te/aTe "S" on effector/target cell conjugate formation

	% target binding cells (mean ± SD)	
	NK cell-enriched suspensions*	Unfractionated suspensions**
- Te/aTe	14.4 ± 1.4	6.9 ± 1.7
+ Te/aTe	3.8 ± 2.0	1.9 ± 0.8
+ Te/aTe "S"	3.3 ± 0.3	3.0 ± 0.0

* Prepared by Percoll gradient separation.

** Plastic non-adherent mononuclear cells.

The percentage of target binding cells was determined by counting the number of lymphocytes bound to target cells and the total number of lymphocytes. At least 400 lymphocytes were counted. Mean ± SD of four experiments.

able Fc sites of the sensitizing antibody (16). In agreement with Kay et al. (16) we found that protein A was without effect on NK cell activity, but when we pretreated the Te/aTe immune complexes with free protein A before incubation with effector cells, the immune complex-induced inhibition of NK cell activity was markedly reduced. This is in accordance with the hypothesis that the immune complex-induced inhibition occurs when the Fc parts of the antigen-antibody complexes are bound to the Fc receptors of NK cells.

Recent data have indicated that the activity of NK cells is subject to considerable regulation. In addition to immune complexes a number of agents including prostaglandins (4), cyclophosphamide (9), azathioprine (21), and corticosteroids (20), are known to depress NK function, while IF (12) and Il-2 (18) among others augment the cytolytic activity of NK cells.

Macrophages and adherent cells produce potent immunosuppressive substances, such as prostaglandins and reactive oxygen (superoxide, hydrogen peroxide, etc.), when stimulated by immune complexes (1). Prostaglandin-mediated inhibition of NK cells has been described (4), and it is possible that reactive oxygen inhibits the NK cells as well (22). Theoretically, the immune complex-induced inhibition of NK cells could be due to prostaglandin production by adherent cells. This is, however, not likely as the same results were obtained with effector cells depleted of adherent cells.

Cell-mediated cytotoxicity involving natural killer cells requires contact between effector and target cells for effective cytolysis. To study whether the immune complexes inhibited the adhesion of effector cells to target structures, single cell agarose assays were performed. We found a markedly decreased number of effector/target cell conjugates, when the effector cells had been incubated with precipitated complexes (Te/aTe) as well as with soluble complexes (Te/aTe "S"), while Sibbitt et al. (23) found that incubation of mononuclear cells with aggregated IgG did not inhibit the adhesion and recognition step, despite concomitant decreases in baseline and interferon-induced NK cell activity. As discussed by Karsch et al. (14), it is possible that antigen

antibody complexes interfere with NK cytotoxicity via steric inhibition of specific cell surface receptors which recognize target structures and are geographically closely situated to Fc receptors. The degree of disturbances of these adjacent membrane receptors might be dependent upon the size and type of immune complexes (8, 14, 17), which would explain the discrepancy between our results and those of Sibbitt et al. (23).

In accordance with Sibbitt et al. (23), who found that aggregated IgG and SLE sera suppressed the baseline as well as the IF enhanced NK activity, we found that neither IF nor Il-2 abolished the immune complex-induced suppression of NK cell activity. IF normally augments NK cell activity by increasing the proportion of NK cells able to bind to the target and by increasing recycling of NK cells (25), and it is therefore understandable that IF had no effect when the binding of effector cells to target cells was inhibited by primary incubation with Te/aTe immune complexes.

The contact between a LGL and a tumour target cell is not only very close, but on occasion the lymphocyte is within the confines of the target cell itself. This penetration of tumour targets by lymphocytes has been termed *emperipolesis* and represents one type of effector/target cell contact leading to cytotoxicity. In consistency with our results, it was shown that aggregated IgG inhibited *emperipolesis* (5).

The demonstration that immune complexes inhibit NK cell activity may be the *in vitro* correlate of patients with immunoinflammatory disease, e.g. systemic lupus erythematosus, who have circulating immune complexes and depressed NK cell activity (10, 14, 23). It is thus possible that immune complexes represent an important mechanism for negative regulation of NK cell activity.

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