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CHARACTERIZATION OF HUMAN LYMPHOID CELL-MEDIATED ANTIBODY-DEPENDENT CYTOTOXICITY (LDAC)

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SUMMARY

Cytotoxicity was studied in a model system using chicken erythrocytes (Ch RBC) labelled with ^{51}Cr as target cells and human peripheral blood lymphoid cells as effector cells. *In vitro* human lymphoid cells are highly efficient in destroying target cells coated with anti-target cell antibody, the mean net percentage cytotoxicity of lymphoid cells from fifty-eight control subjects being $64{\cdot}27 \pm 2{\cdot}06$ (SEM). In the absence of antibody the mean net percentage cytotoxicity was $6{\cdot}90 \pm 0{\cdot}46$. As little as 10 ng rabbit anti-Ch RBC IgG was required to cause significant target cell lysis. Studies on the nature of the lymphoid cell-dependent cytotoxic antibody showed that it is localized in the 7S IgG region of whole serum and that an intact Fc region is required; the $F(ab')_2$ fragment obtained by pepsin digestion of IgG was inactive although able to inhibit the cytotoxic activity of the whole undigested IgG. Investigation of the kinetics of LDAC showed that when antibody was added to the final culture medium target cell lysis progressed rapidly (detectable within 2 hr) and linearly with time up to 8 hr. Thereafter the rate of lysis decreased reaching a maximum after 12 hr culture. With cultures containing target cells which have been pre-incubated with antibody, lysis occurred even more rapidly, detectable within 30 min and reaching a maximum after only 3–4 hr culture. The maximum cytotoxicity in this system was, however, lower than that obtained when antibody was added directly to the culture medium. Cytotoxicity could be inhibited by the addition of aggregated human IgG, as little as 5 μg causing 100% inhibition of target cell lysis. Study of the nature of the effector lymphoid cell showed, first, that viable cells were required, twice frozen/thawed lymphoid cell suspensions being inactive; secondly, that active protein synthesis by the effector cell was not an essential prerequisite, pretreatment of lymphoid cells with mitomycin C having no significan effect on their ability to lyse antibody-coated target cells but significantly reducing their ability to transform in response to the mitogen PHA; thirdly, that the effector cell is non-phagocytic, non-plastic or glass-adherent and does not bear sur-

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face immunoglobulin; and fourthly that significant cytotoxicity is detectable even with a total lymphoid cell to target cell ratio of 1:2.

INTRODUCTION

During the past decade considerable evidence has accumulated that lymphoid cells *in vitro* are capable of destroying appropriate target cells. These cytotoxic effects of lymphoid cells have been assumed to reflect their effector role in tissue-damaging immune responses such as occur in allograft rejection, tumour surveillance and in certain autoimmune diseases (Perlmann & Holm, 1969). At least two distinct pathways appear to be involved in the production of immunologically specific cell damage. One of these mechanisms involves lymphoid cells with intrinsic specificity for target cell antigens. Lymphoid cells of this type are thought to be thymus-dependent (Cerrottini, Nordin & Brunner, 1970a, b; Goldstein *et al.*, 1972). The second mechanism which has received much attention in recent years requires the participation of lymphoid cells and antibody. The cytotoxic cell in this situation has no direct specificity for target cell antigens but is activated by antibody complexed to the target cell antigen. Furthermore, these effector cells can develop in the absence of thymic influence (MacLennan & Harding, 1970a; Harding *et al.*, 1971; Van Boxel *et al.*, 1972; Greenberg *et al.*, 1973a). The concept of LDAC was first recognized by Möller in 1965 and described more fully by Perlmann & Holm (1968). These latter authors studied the effect of spleen cells from both control and BCG immunized guinea-pigs on the release of isotope from Ch RBC coated with PPD and noted that while spleen cells from immunized animals were cytotoxic to these target cells, similar levels of cytotoxicity could be achieved with lymphoid cells from non-immunized donors in the presence of serum from immunized animals.

Since these early experiments LDAC has been described in numerous experimental systems (Lundgren, Collste & Möller, 1968; MacLennan & Loewi, 1968; Perlmann & Holm, 1969; Wassermann *et al.*, 1969; MacLennan, Loewi & Harding, 1970; MacLennan & Harding, 1970b; Bubenik, Perlmann & Hasek, 1970; Möller, 1970; Wunderlich, Rosenberg & Connelly, 1972). Target cells have included chicken, duck, sheep or burro erythrocytes, Chang liver cells, mouse L cells and mouse mastocytoma cells; antibody has been directed against natural target cell surface antigens or against antigens coupled to the target cell surface such as PPD or thyroglobulin and effector lymphoid cells have been obtained from peripheral blood, lymph node or spleens from man or animals including rats, mice, chickens and guinea-pigs. The purpose of this paper is to investigate, using a single well-defined model system, some of the basic characteristics of this cytotoxic mechanism.

MATERIALS AND METHODS

(i) *Preparation of target cells*

Blood was routinely collected from the wing vein of White Leghorn chickens aged between 6 and 12 weeks. Five millilitres was collected daily, heparinized (Evans, 10 u/ml), washed three times in phosphate-buffered saline (PBS), pH 7·2 and adjusted to approximately 2×10^8 cells/ml. A hundred microlitres of Ch RBC were then incubated with 100 μCi ^{51}Cr (Amersham) for 1 hr at 37°C. The labelled cells were then washed once in PBS and divided into two aliquots, each of 100 μl. One aliquot was incubated with PBS (100 μl), the second with 100 μl of 10^{-2} diluted rabbit anti-Ch RBC serum (heat-inactivated 56°C/1 hr). After 60 min incubation at 37°C, the target cells were washed three times in PBS, counted, pelleted

and kept at room temperature until required (up to 2 hr). Immediately prior to use, the labelled cells were washed twice in Eagle's medium (Wellcome Reagents Ltd) supplemented with 10% heat-inactivated foetal calf serum (FCS, Wellcome Reagents Ltd) and adjusted to 10^6 cells/ml. In some experiments target cell antibody was added to the culture medium directly in which case the target cells were labelled with ^{51}Cr and then washed as described above. Sheep red blood cells were prepared and labelled in an identical manner. Rabbit anti-sheep RBC serum (Wellcome Reagents Ltd) was used at the pre-incubation stage.

(ii) *Preparation of lymphoid cells*

Lymphoid cells were prepared from peripheral blood of healthy donors. These consisted of medical and laboratory personnel and BTS blood donors. Twenty-five to 50 ml heparinized blood was collected (Evans, 10 u/ml) and lymphoid cells separated by density gradient centrifugation on Ficoll–Triosil exactly as described by Calder *et al.* (1973). Unless otherwise stated, the prepared lymphoid cells were resuspended in Eagle's + 10% FCS to a concentration of approximately 2×10^6/ml and were incubated in a flat tissue culture flask (Falcon Plastics) for 2 hr at 37°C. Non-adherent lymphoid cells were then washed twice in Eagle's + 10% FCS and adjusted to 10^6 cells/ml.

(iii) *Cytotoxic assay procedure*

Cultures were set up in triplicate in LP3 culture tubes (Luckhams Ltd) containing lymphoid cells (4×10^5) and target cells (2×10^4) in a total volume of 400 μl culture medium. One group contained target cell antibody, either pre-incubated with the target cells or added directly to the culture medium, the second group contained no antibody. Control cultures were also set up containing washed unlabelled Ch RBC in place of lymphoid cells. Maximum isotope release was estimated by adding target cells to cultures containing 1/10 guinea-pig serum as a source of complement. Cultures were incubated at 37°C for 18 hr in air: 5% CO_2 and then centrifuged at 200 *g* for 10 min. 200 μl supernatant was removed and counted in an automatic well-type gamma counter (Wallac).
Calculations were as follows:

$$\text{(I) Percentage cytotoxicity} = \frac{\text{mean total supernatant count}}{\text{total activity}} \times 100.$$

$$\text{(II) Net \% lymphoid cell cytotoxicity} = \text{\% lymphoid cell cytotoxicity} - \text{\% spontaneous RBC release.}$$

$$\text{(III) Cytotoxic index} = \frac{\text{net \% lymphoid cell cytotoxicity}}{\text{\% max isotope release} - \text{\% spontaneous release}} \times 100.$$

(iv) *Preparation and fractionation of anti-target cell antibody*

Antiserum to Ch RBC was raised in rabbits following three intramuscular injections of 0·2 ml packed Ch RBC emulsified in Freund's complete adjuvant at 21-day intervals. Rabbit anti-Ch RBC serum was separated into 19S, 7S and albumin components by column chromatography using Sephadex G-200.

(v) *Preparation of the $F(ab')_2$ fragment of rabbit anti-Ch RBC IgG*

Rabbit anti-Ch RBC IgG was prepared by ion exchange fractionation using DE52 cel-

lulose (Whatman). The $F(ab')_2$ fragment was prepared by pepsin digestion exactly as described by Stanworth & Turner (1973).

(vi) *Preparation and aggregation of human IgG*

Human IgG was prepared from pooled normal human plasma by a batch DEAE–cellulose technique (James, 1969). The prepared IgG was heat aggregated as described by Hallberg (1974). Briefly human IgG (40 mg/ml in Tris–Earle buffer, pH 7·2) was heated at 63°C for 15 min and centrifuged at 150 *g* for 10 min to remove gross precipitates. The supernatant was diluted in PBS to give a protein concentration of 1 mg/ml and was then stored in small aliquots at −20°C.

(vii) *PHA-transformation test*

The ability of human peripheral blood lymphocytes to respond to the mitogen PHA was performed exactly as described by Penhale *et al.* (1974).

(viii) *Identification of T and B lymphocytes and phagocytic cells*

T cells were identified by sheep cell rosettes. The method used was that of Jondal, Holm & Wigzell (1972) as modified by Stjernsward *et al.* (1973) and described in detail by Urbaniak, Penhale & Irvine (1973). B cells were identified by surface immunoglobulin. An indirect fluorescence method (Jondal *et al.*, 1972) as described by Urbaniak *et al.* (1973) was employed using rabbit anti-IgG (Fab) followed by FITC conjugated goat anti-rabbit IgG serum. Phagocytic cells were identified by uptake of Neutral Red.

(ix) *Preparation of anti-Ig–Ig columns*

Sepharose 2B (Pharmacia) was activated by the cyanogen bromide method described by Cuatrecasas (1970). Briefly, 20 ml washed Sepharose 2B were mixed with approximately 4 g cyanogen bromide and the mixture brought to pH 11 by the addition of 2 M NaOH and maintained at this pH until the activation was complete (10–12 min). The activated Sepharose was then washed in 50 volumes of 0·1 M $NaHCO_3$ on a sintered glass filter funnel and resuspended in a minimum volume of the same buffer. An excess of human IgG (900 mg) was added and conjugation allowed to proceed with continuous rotation for 72 hr at 4°C. Mini-columns adapted from glass pasteur pipettes were packed with 2 ml of IgG-conjugated Sepharose, washed with Eagle's medium and kept at 4°C. Two millilitres of 1/4 diluted anti-IgG (Fab) (Behringwerke) was then added, the column sealed and allowed to equilibrate for 2 hr at 4°C. Immediately prior to use, the excess unbound anti-IgG (Fab) was washed off with 10 ml Eagle's medium. Twenty-five to 50×10^6 lymphoid cells were then applied to the column in a volume of 1–2 ml Eagle's and the eluted cells passed through the column twice. The finally recovered cells were washed three times in Eagle's + FCS and used immediately in the cytotoxic assay.

(x) *Preparation of glass and plastic bead columns*

Degalan V26 plastic beads were washed repeatedly in distilled water and packed into a column 0·9 × 15 cm (Pharmacia) with a disc of nylon mesh as filter. The beads were then washed in 5 volumes of Eagle's + FCS and allowed to equilibrate at 37°C for 30 min. Twenty-five to 30×10^6 lymphoid cells in a volume of 2 ml of Eagle's + FCS were applied to

the column which was then incubated horizontally for 30 min at 37°C. Cells were eluted in 20 ml Eagle's + FCS, washed three times in culture medium and used immediately.

Glass bead columns (0·2 mm diameter) were prepared and used in an identical manner.

RESULTS

Effect of human lymphoid cells on antibody-coated erythrocytes

Fig. 1 shows the effect of lymphoid cells from fifty-eight normal control subjects on the release of isotope from Ch RBC pre-incubated with either PBS or anti-Ch RBC antibody.

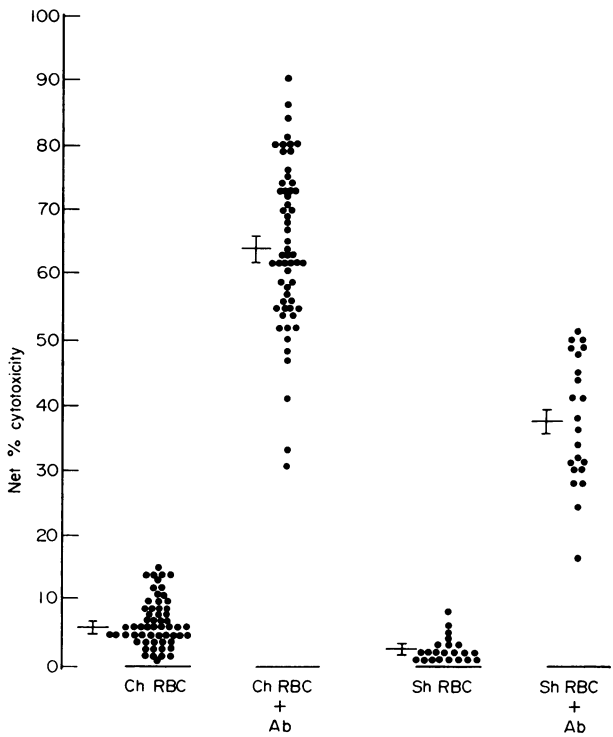

FIG. 1. The effect of peripheral blood lymphoid cells on the release of isotope from chicken or sheep erythrocytes pre-incubated with either buffer or specific anti-target cell antibody.

The addition of lymphoid cells to target cells pre-incubated with buffer gave a mean net % isotope release of 6·90 ± 0·46 ranging from 1 to 15%. The addition of lymphoid cells to antibody-coated Ch RBC caused a mean net % isotope release of 64·27 ± 2·06, ranging from 31 to 90%. In the absence of lymphoid cells, the spontaneous release of isotope from target cells pre-incubated with either buffer or antibody was routinely between 1 and 3%. Fig. 1 also shows the results of an identical series of experiments studying the effect of lymphoid cells from twenty-two control subjects on the release of isotope from sheep erythrocytes pre-incubated with either buffer or anti-Sh RBC antibody. In the absence of antibody, lymphoid cells exerted very little cytotoxic activity, the mean net % isotope release being 2·45 ± 0·39, ranging from 1 to 8%. In the presence of antibody, lymphoid cells caused significant lysis,

the mean net % cytotoxicity being 37·54±2·11, ranging from 16 to 51%. In the absence of lymphoid cells, the spontaneous isotope release from Sh. RBC in the presence or absence of antibody was between 8 and 10%.

Effect of antibody dilution on cytotoxicity

Fig. 2 shows the effect of lymphoid cells from eight control subjects on the release of isotope from Ch RBC cultured with varying dilutions of antibody ranging from 5×10^{-4} to 5×10^{-8} anti-Ch RBC serum. At low antibody dilutions, $1{\cdot}25 \times 10^{-6}$ to 5×10^{-8}, cytotoxicity was low, just reaching significance at $1{\cdot}25 \times 10^{-6}$ when the cytotoxic index was 10·8±5·1 ($P \leqslant 0{\cdot}01$). Increasing antibody concentration to 5×10^{-6}, $1{\cdot}25 \times 10^{-5}$ and 5×10^{-5} caused a

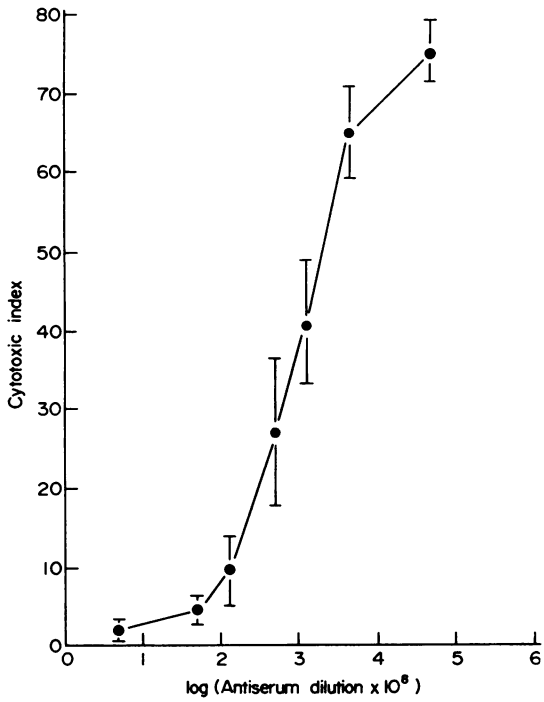

FIG. 2. The effect of antibody concentration on antibody dependent lymphoid cell mediated cytotoxicity.

linear increase in cytotoxicity, the cytotoxic indices being 26·8±9·5, 40·6±8·3 and 64·6±6·6 respectively. At the highest antibody concentration of 5×10^{-4} the cytotoxic index increased to 75·4±4·4.

The effect of total lymphoid cell concentration on cytotoxicity

Fig. 3 shows the effect of varying lymphoid cell numbers from seventeen control subjects on the release of isotope from Ch RBC in culture with a fixed concentration of antibody (5×10^{-5} anti-Ch RBC serum). A linear relationship between total lymphoid cell numbers and cytotoxicity was observed using lymphoid: target cell ratios of 5:1, 2·5:1, 1·25:1 and 1:2, the corresponding cytotoxic indices being 73·4±5·4, 58·0±6·5, 35·9±5·1 and 14·3±2·2.

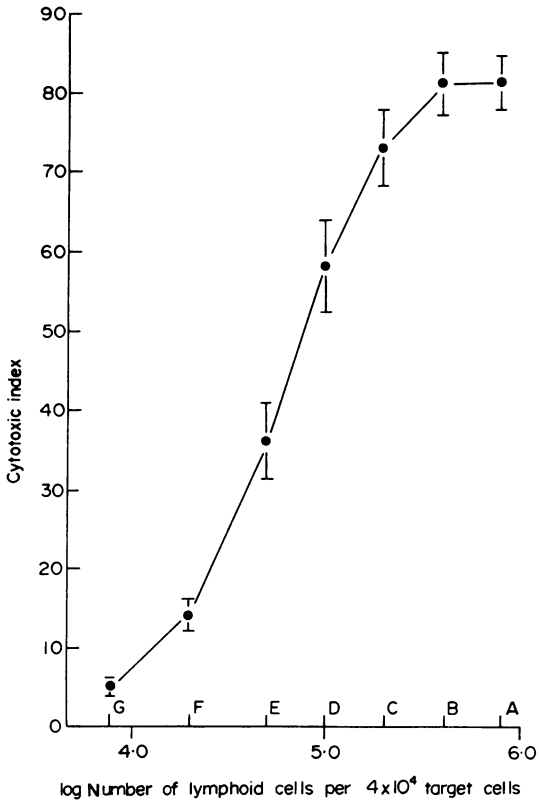

FIG. 3. The effect of lymphoid cell concentration on antibody-dependent cytotoxicity.

A	8×10^5 lymphoid cells,	4×10^4 target cells,	ratio 20:1.
B	4×10^5 lymphoid cells,	4×10^4 target cells,	ratio 10:1.
C	2×10^5 lymphoid cells,	4×10^4 target cells,	ratio 5:1.
D	10^5 lymphoid cells,	4×10^4 target cells,	ratio 2·5:1.
E	5×10^4 lymphoid cells,	4×10^4 target cells,	ratio 1·25:1.
F	4×10^4 lymphoid cells,	8×10^4 target cells,	ratio 1:2.
G	$2{\cdot}4\times10^4$ lymphoid cells,	$1{\cdot}2\times10^5$ target cells,	ratio 1:5.

Using greater total lymphoid cell numbers, i.e. ratios of 10:1 and 20:1, did not greatly increase cytotoxicity, the indices being $81{\cdot}2\pm4{\cdot}4$ and $81{\cdot}41\pm3{\cdot}6$. At the lowest lymphoid: target cell ratio of 1:5, cytotoxicity was not significant, the index being $4{\cdot}76\pm0{\cdot}78$.

Kinetics of LDA cytotoxicity

Fig. 4(a) shows the results of three replicate experiments studying the relationship between cytotoxicity and time. In these experiments cultures were set up containing optimum concentrations of both lymphoid cells and antibody, i.e. lymphoid:target cell ratio of 10:1 and antibody concentration of 5×10^{-5} anti-Ch RBC serum. Cytotoxicity was measured at hourly intervals up to 12 hr and then at 20 hr. Significant lysis of target cells was detectable within 2 hr and progressed linearly with time for 8 hr. Thereafter cytotoxicity increased only slowly. The cytotoxic indices observed after 20 hr were slightly but not significantly greater

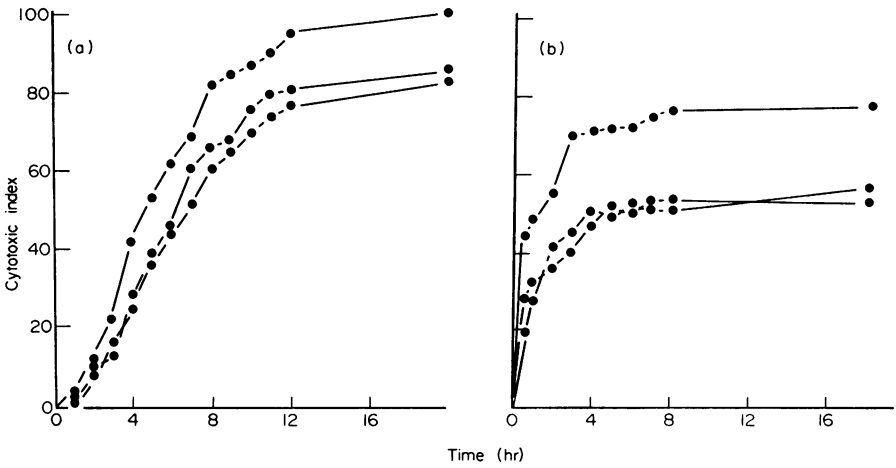

FIG. 4. Time-course of the cytotoxic effect of lymphoid cells on Ch RBC in the presence of anti-Ch RBC antibody. (a) Antibody present in culture medium; (b) target cells pre-incubated with antibody.

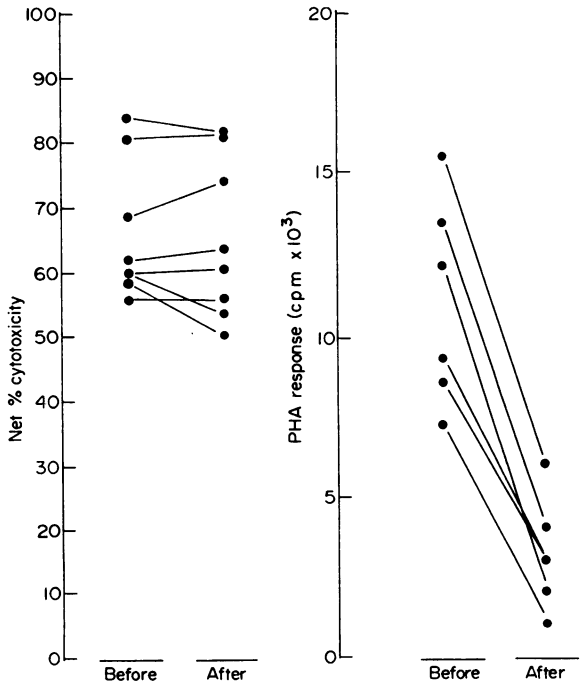

FIG. 5. The effect of pretreatment of lymphoid cells with mitomycin C on their ability to respond to PHA and to lyse antibody-coated chicken erythrocytes.

than those observed after 12 hr culture. Fig. 4(b) shows the rate of target cell lysis in cultures containing lymphoid cells and target cells which had been pre-incubated with a 1/100 dilution of anti-target cell antiserum and then washed. The lymphoid cell:target cell ratio was still 10:1. Under these conditions, target cell lysis progressed very rapidly and was detectable within 30 min reaching a maximum after only 3–4 hr.

Factors which influence LDA cytotoxicity

(i) *Viability of lymphoid cells.* Lymphoid cells which were dead following repeated freezing and thawing were unable to destroy antibody-coated Ch RBC.

(ii) *Mitomycin C.* Fig. 5 shows the effect of pretreatment of lymphoid cells with mitomycin C (25 μg/ml) on their ability to respond to PHA and to destroy antibody-coated Ch RBC. The mean net % cytotoxicity of untreated lymphoid cells on antibody-coated Ch RBC in eight replicate experiments was $66{\cdot}00 \pm 3{\cdot}93$. The corresponding cytotoxicity of mitomycin-treated lymphoid cells was $65{\cdot}25 \pm 4{\cdot}36$. Thus pretreatment of lymphoid cells with mitomycin C did not significantly impair their ability to mediate antibody-dependent cytotoxicity. In contrast, pretreatment of lymphoid cells with mitomycin C significantly reduced their ability to transform in response to the mitogen PHA. The mean uptake of tritiated thymidine by untreated lymphoid cells was $13{\cdot}16 \pm 1{\cdot}49$ ct/min $\times 10^3$ whereas the uptake by mitomycin-treated lymphoid cells was only $3{\cdot}16 \pm 0{\cdot}70$ ct/min $\times 10^3$.

(iii) *Aggregated human IgG.* The inhibitory effect of aggregated human IgG on LDA cytotoxicity was studied and the results are shown in Table 1. Using an optimum concentration of anti-target cell antibody (5×10^{-5} anti-Ch RBC serum) and an excess of lymphoid cells (lymphoid:target cell ratio of 20:1) aggregated IgG (100, 50 and 5 μg) had no significant inhibitory effect. With optimum concentrations of both lymphoid cells (ratio 5:1) and antibody, significant inhibition of cytotoxicity was observed with 100 and 50 μg IgG. Under these conditions, 5 μg aggregated IgG gave variable results, inhibition ranging from 3 to 47%. With a lower dilution of antibody (5×10^{-6} anti-Ch RBC serum) and a lymphoid:target cell ratio of 20:1, aggregated IgG, 100 and 50 μg caused almost 100% inhibition, but again the inhibition with 5 μg was variable, ranging from 5 to 59%. Maximum sensitivity under the conditions tested was obtained using an antibody dilution of 5×10^{-6} and a lymphoid:target cell ratio of 5:1 when all three concentrations of aggregated human IgG caused nearly 100% inhibition.

Characterization of the cytotoxic antibody

(i) *Localization in whole serum.* Fig. 6 shows the effect of lymphoid cells on the release of isotope from Ch RBC preincubated with the 19S, 7S, and albumin fractions of rabbit anti-Ch RBC serum. The addition of lymphoid cells to target cells pre-incubated with either whole serum or the 7S IgG fraction of whole serum caused significant cytotoxicity, the mean net % isotope release being $42{\cdot}75 \pm 3{\cdot}02$ and $50{\cdot}75 \pm 2{\cdot}53$ respectively. In contrast, lymphoid cells had no significant cytotoxicity on target cells pre-incubated with either the 19S IgM or 4·5S albumin components of whole serum, the mean net % isotope release being $4{\cdot}25 \pm 1{\cdot}62$ and $3{\cdot}75 \pm 1{\cdot}53$ respectively.

(ii) *Cytotoxic activity of the $F(ab')_2$ fragment of rabbit anti-Ch RBC IgG.* The results of three replicate experiments studying the effect of lymphoid cells on Ch RBC in the presence of rabbit anti-Ch RBC IgG or its $F(ab')_2$ fragment are shown in Table 2. Whole undigested IgG caused significant cytotoxicity when added in concentrations of 1000, 100 and 10 ng.

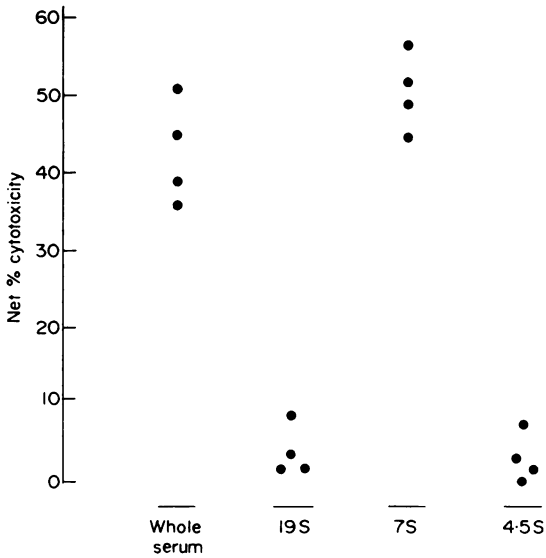

FIG. 6. The lymphoid cell-dependent cytotoxic activity of the 19S, 7S and 4·5S components of anti-target cell antiserum.

TABLE 1. The inhibitory effect of aggregated human IgG on LDA cytotoxicity

Antibody DIL [n]	Ag IgG (μg/culture)	Inhibition in replicate expts (%) 20:1*	5:1*
5×10^{-5}	100	3, 15	57, 100, 98, 98
	50	0, 6	43, 97, 92, 82
	5	4, 0	3, 47, 23, 19
5×10^{-6}	100	100, 100, 100	100, 100
	50	92, 84, 93	100, 100
	5	53, 5, 59	95, 88, 96, 89

* Lymphoid cell: target cell ratio (2×10^4 target cells).

TABLE 2. Cytotoxic effect of lymphoid cells on Ch RBC in the presence of rabbit anti-Ch RBC IgG and its $F(ab')_2$ fragment

Experiment number	% Cytotoxicity IgG* 1000	100	10	1	$F(ab')_2$* 1000	100	10	1
1	82	84	50	4	2	3	1	0
2	81	70	28	3	0	0	0	0
3	88	73	28	0	0	0	0	0

* Concentration in ng of IgG and $F(ab')_2$ per culture.

TABLE 3. Cytotoxic effect of lymphoid cells before and after passage through plastic or glass bead columns

Column	% Cytotoxicity		% Fluorescence		% Phagocytes	
	Before	After	Before	After	Before	After
Plastic	64	79	22	22	20	4
	69	79	23	18	10	2
	42	51	15	16	7	1
	61	62	20	23	6	2
Glass	45	65	15	13	16	1
	59	61	6	10	10	2
	47	58	16	14	8	<1
	75	79	17	18	11	2

The $F(ab')_2$ fragment obtained by pepsin digestion of IgG caused no cytotoxicity even at the highest concentration of 1000 ng. The $F(ab')_2$ fragment did, however, cause inhibition of the IgG induced cytotoxicity. In one experiment the addition of 1000 ng $F(ab')_2$ to cultures containing 1000 ng IgG reduced the % cytotoxicity from 91·0 to 65·0.

Characteristics of the effector lymphoid cell

(i) *Surface adherence and phagocytic properties.* Lymphoid cells before and after incubation for 30 min on glass or plastic bead columns were analysed for: (a) the number of cells bearing surface Ig; (b) the number of phagocytic cells; and (c) the ability to lyse antibody-coated Ch RBC. Results of four replicate experiments are summarized in Table 3. Incubation of lymphoid cells on glass or plastic for 30 min significantly reduces the number of phagocytic cells but does not significantly alter either the number of cells bearing surface Ig or their cytotoxic effect on antibody-coated Ch RBC.

(ii) *Cytotoxic activity of lymphoid cells depleted of the surface Ig-bearing cell population.* In four replicate experiments lymphoid cells were passed through a human-Ig–anti-Ig coated column. The number of Ig-bearing cells before and after passage through the column and the cytotoxic capacity of both untreated and column passaged lymphoid cells is shown in Table 4. In all four experiments, passage of lymphoid cells through the anti-immunoglobulin column caused a dramatic reduction in the number of Ig-bearing cells. The removal

TABLE 4. Cytotoxic activity of lymphoid cells before and after removal of Ig-bearing lymphocytes by passage through an anti-immunoglobulin column

Number	% Cytotoxicity		% Fluorescence	
	Before	After	Before	After
1	57	27	23	3
2	60	53	10	1
3	57	48	16	<1
4	59	62	18	<1

of Ig-bearing cells from peripheral blood lymphoid cell suspensions had no significant effect on their antibody-dependent cytotoxic activity except in one sample where cytotoxicity was reduced by almost 50%.

DISCUSSION

Previous studies on the cytotoxic effect of human peripheral blood lymphoid cells on antibody-coated Ch RBC have been performed with relatively small numbers of lymphoid cell samples and absolute figures for the percentage isotope release in the presence or absence of antibody are rarely quoted. Fig. 1 gives some indication of the variation in the cytotoxic activity of lymphoid cells from fifty-eight control subjects. The % isotope release from Ch RBC in the presence of anti-Ch RBC antibody is consistently and significantly higher than that observed in the absence of antibody but the increase varied from a minimum of five-fold to a maximum of eighteen-fold. Sheep erythrocytes were also used as target cells but proved to be significantly less susceptible to lymphoid cell mediated cytolysis. One of the advantages of using erythrocytes as target cells is their low spontaneous isotope release, rarely exceeding 10% over 24–48 hr culture. Tissue cells such as Chang liver cells or mouse fibroblasts have a much higher spontaneous rate of isotope release often as great as 30% during 24–48 hr culture.

We have found that when antibody is added to the culture medium cytotoxicity occurs rapidly and linearly with time up to 8 hr, reaching a maximum after 12 hr culture and requires only a very low concentration of target cell antibody. As little as 10 ng rabbit anti-Ch RBC IgG caused significant lysis. These results are somewhat different from those reported by Perlmann, Perlmann & Wigzell (1972) who also using Ch RBC as target cells, found lysis to occur most rapidly within the first 5 hr, thereafter decreasing and reaching a maximum after 20–40 hr culture. Möller & Svehag (1972) using sheep fibroblasts as target cells found cytotoxicity to proceed linearly with time but very slowly, only reaching a maximum after 4 days of culture. We also found that the addition of lymphoid cells to target cells which had been pre-incubated in antibody and then washed resulted in a highly significant degree of lysis within 30 min. Maximum target cell lysis was observed after only 3–4 hr and did not increase significantly even after 20 hr culture. This more rapid rate of lysis was not unexpected since the antibody was already available on the target cell surface. The maximum cytotoxicity observed in this system was, however, lower than that obtained when antibody was added directly to the culture medium. The significance of this difference is uncertain but probably reflects differences in the methodology of the two procedures.

Further evidence for the efficiency of this *in vitro* cytotoxic mechanism comes from the finding that relatively few lymphoid cells are required to cause significant target cell lysis. Maximum isotope release is observed with a total lymphoid cell:target cell ratio of 10:1 but significant cytotoxicity can still be obtained with a ratio of 1:2. If it is postulated that the effector lymphoid cell belongs to neither the T- nor the B-lymphocyte population, it follows that a peripheral blood lymphoid cell suspension can contain a maximum of 5–15% cytotoxic K cells and therefore each K cell has the capacity to destroy several target cells. MacLennan (1972) has studied the effect of total lymphoid cell numbers on the release of isotope from antibody-coated Chang liver cells. Those target cells would appear to be considerably more resistant to lysis than Ch RBC, maximum isotope release only being observed with a lymphoid:target cell ratio of $>100:1$. With an excess of target cells to

lymphoid cells, cytotoxicity was not detectable. Similarly, Möller & Svehag (1972), using sheep fibroblasts as target cells, found cytotoxicity to be optimum with a fifty-fold excess of lymphoid cells.

The lymphoid cell-dependent cytotoxic antibody is localized exclusively in the 7S region of Sephadex G-200-separated whole serum and an intact Fc region is essential to initiate cytotoxicity. The $F(ab')_2$ fragment of the antibody was itself inactive although able to inhibit the cytotoxic activity of the whole antibody molecule. These characteristics are similar to those previously reported for anti-Chang cell antibody (MacLennan, Connel & Gotch, 1974), anti-sheep fibroblast antibody (Möller & Svehag, 1972) and for anti-Ch RBC antibody (Larsson & Perlmann, 1972).

As shown in Fig. 5, active protein synthesis by the effector lymphoid cell is not essential for the manifestation of cytotoxicity and provides further evidence that the mechanism of target cell destruction is essentially different from that exerted by specifically sensitized T lymphocytes. Möller & Svehag (1972) have previously shown that mitomycin C has no effect on the ability of human lymphoid cells to destroy antibody-coated sheep fibroblasts during 3–4 days culture. In this study the PHA responsiveness of the mitomycin-treated lymphoid cells was studied concurrently and found to be significantly reduced, thus indicating indirectly that protein synthesis was in fact depressed.

As expected, aggregated human IgG caused significant inhibition of LDA cytotoxicity, presumably through competition for the Fc receptor on the effector lymphoid cell. Sensitivity to inhibition is markedly increased when sub-optimal concentrations of both lymphoid cells and antibody are used. Under these conditions as little as 5 μg aggregated human IgG caused 100% inhibition of target cell lysis. An earlier report of Hallberg (1974) found that very much higher concentrations of aggregated IgG were required to cause inhibition of cytotoxicity (5000 and 500 μg). This was possibly due to the fact that a relatively large excess of lymphoid cells to target cells was used.

Our investigation of the nature of the effector cell indicates that it is non-phagocytic and that it adheres to neither glass nor plastic. These latter findings may not be conclusive since it proved difficult to recover the adherent cell population to test its cytotoxic capacity. However, the human peripheral blood effector cell does appear to differ from the effector cell in mouse spleen cell suspensions which, as described by Greenberg, Shen & Roitt (1973b), adheres strongly to plastic.

The results of the antibody-dependent cytotoxic activity of human lymphoid cells depleted of the Ig-bearing lymphocyte population by passage through a human Ig–anti-Ig column suggested that the effector lymphoid cell is non-immunoglobulin bearing. Perlmann *et al.* (1972) using an identical method for the removal of Ig-bearing cells found that the depleted cell populations were unable to lyse antibody-coated target cells but suggested that the loss of activity could have been due to the removal of K-cells by combination with the activated Fc region of the human Ig–anti-Ig complex on the column. Current experiments of Wigzell *et al.* (1974) have further shown that the effector human cytotoxic cell is not retained on a column coated with human Ig $(Fab')_2$–anti-Ig. Similar results for the effector cell in mouse spleen cells have been reported (Greenberg *et al.*, 1973b).

More direct evidence that the effector cell is non-B is the finding by Wisloff & Froland (1973) that patients with severe hypogammaglobulinaemia have normal K-cell function. Similarly, Calder *et al.* (1974) have shown that bursectomized chickens also have a normal K-cell response.

The precise origin of the effector lymphoid cell in both man and animals remains to be established. It may belong to a subpopulation of non-immunoglobulin-bearing B lymphocytes, or, as Greenberg *et al.* (1973b) have suggested, it may be an immature non-phagocytic monocyte. Alternatively, the K cell may belong to an as yet unidentified subpopulation of lymphocytes.

In vitro the K cell has a considerable cytotoxic potential but its precise biological role *in vivo* remains open to speculation. The possibility that this cytotoxic mechanism is involved in the rejection of foreign cells or in the production of autoallergic tissue damage has not been established but it would seem likely that such a highly efficient cytotoxic mechanism is of great biological significance.

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