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Identity of effector cells participating in the reverse antibody-dependent cell-mediated cytotoxicity

R. K. SAXENA, QUEEN B. SAXENA & W. H. ADLER *Immunology Section, GRC, National Institute on Aging, National Institutes of Health, Baltimore, Maryland, U.S.A.*

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Summary. Our previous work has shown that antibody-coated mouse spleen cells express enhanced cytotoxic activity against some Fc-receptor-bearing target tumour cells by a mechanism which appears to be similar to an antibody-dependent cell-mediated cytotoxicity (ADCC) reaction with reversed polarity of the antibody bridge (R-ADCC). In this report we have shown that (i) the levels of basal natural killer (NK), ADCC and R-ADCC cytotoxic activities in mouse spleen cells are strongly correlated with each other, (b) simultaneous induction of ADCC and R-ADCC reactions does not result in an additive cytotoxic response, and (iii) YAC cells which do not bear Fc receptors and are highly sensitive to lysis by NK cells, can specifically and competitively inhibit the ADCC and R-ADCC reactions. These results suggest that the R-ADCC reaction may be mediated by the same effector cell population as mediates NK and ADCC reactions against tumour target cells.

INTRODUCTION

In the mouse system, a population of killer lymphocytes (K cells) distinct from conventional T or B cells mediates antibody-dependent cell-mediated cytotoxicity (ADCC) against antibody-sensitized tumour cells (Reviewed by Pearson, 1978). Recently we have described a new kind of antibody-dependent cytotoxicity in mouse spleen cells, whereby the treatment of effector cell preparations with specific anti-H-2 antibodies could enhance their cytotoxicity against a human leukaemia cell line K562 (Saxena & Adler, 1979). Effector cells responsible for the alloantibody-induced cytotoxicity were distinct from conventional T cells, B cells or macrophages (Saxena & Adler, 1979; Saxena, Saxena & Adler, 1980). These results have recently been confirmed by other investigators (Brunda, Herberman & Holden, 1981). Our further work has indicated that the cytotoxicity enhancement by alloantibodies is not mediated by interferon, nor does it appear to be a non-specific effect of membrane perturbation induced by membrane-bound antibodies (Saxena, Adler & Nordin, 1981a). A distinguishing feature of the effect of alloantibody was that the enhancement of cytotoxicity could only be observed against certain Fc-receptor-positive target cell lines (Saxena *et al.*, 1981a; Saxena, Saxena & Adler, 1981b). In addition, an intact and free Fc region of the effector cell bound alloantibodies was crucial to the demonstration of alloantibody-induced cytotoxicity (Saxena *et al.*, 1981b). On the basis of these results we proposed that the bridge formed by the alloantibody attached to effector cells on the one side and interacting with Fc receptors on the target cells on the other could

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; LU, lytic units; NK, natural killer; R-ADCC, reverse ADCC.

Correspondence: Dr R. K. Saxena, National Institute on Aging, NIH, 4940 Eastern Avenue, Baltimore, Maryland 21224, U.S.A.

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reinforce and facilitate the effector–target interaction resulting in an augmented cytotoxicity (Saxena *et al.*, 1981b). The phenomenon thus appears to be similar to the classical ADCC with the important difference of a reversal of the direction of antibody molecules during an effector–target interaction. For this reason, we referred to the phenomenon of alloantibody-induced cytotoxicity as reverse ADCC (Saxena *et al.*, 1981b); this term will continue to be used in the present communication. Reverse-ADCC (R-ADCC) represents a novel way in which antibody can influence the cytotoxic effector–target cell interaction. The biological significance of R-ADCC if any, is not known, the phenomenon may however be potentially important in certain real biological situations (Saxena, 1982). It is not known whether K cells, natural killer (NK) cells or yet another cell type participate in R-ADCC as effector cells. In the present study attempts were made to explore the identity of effector cells responsible for R-ADCC. On the basis of the experimental evidence presented, it appears that a single population of lymphocytes may be responsible for natural killer, antibody-dependent and reverse antibody-dependent cell-mediated cytotoxicity activities.

MATERIALS AND METHODS

Animals

Six to 16 week old female C57BL/6 mice used in this study were obtained from the Jackson Laboratories, Bar Harbor, Maine.

Antisera

Anti-H-2^b antiserum was prepared by repeated intraperitoneal (i.p.) injections of C57BL/6 (H-2^b) spleen cells into DBA/2 mice. Anti-K562 antiserum was raised by hyperimmunization of C57BL/6 mice with tissue cultured K562 tumour cells which were washed several times and suspended in normal saline before use. Anti-NK-1 antiserum [(C3H × BALB/c) F_1 *v.* CE antiserum] which reacts with a non-H-2 antigen selectively expressed on less than 5% of the C57BL/6 mouse spleen cells (Glimcher, Shen & Cantor, 1977), was kindly provided by Dr Fung-Win Shen of the Memorial Sloan-Kettering Cancer Center, New York.

Tumour cell lines and ^{51}Cr release assay

The human leukaemia cell line K562 and the mouse lymphoma YAC cell line were propogated as suspension cultures in RPMI 1640 medium with 10% heat inactivated foetal calf serum (FCS). K562 target cells were labelled with ^{51}Cr by incubating 2×10^6 target cells in 150 μl tris-buffered saline (pH 7·4) with 200 μl of radioactive sodium chromate (^{51}Cr, 1 mCi/ml) at 37° for 30 min. Labelled cells were washed twice and suspended in RPMI 1640 with 10% FCS at a density of 1×10^5 cells per ml. Required numbers of effector spleen cells along with 10^4 ^{51}Cr-labelled target cells were placed in wells of a 96-well round bottom microtitre plate (Falcon Products, Oxnard, Calif.). The total incubation medium volume (RPMI 1640 + 10% FCS) in each well was 0·2 ml. Plates were centrifuged at 400 ***g*** for 3 min and incubated at 37° for 4 or 20 hr. After incubation, 0·1 ml of supernatant was removed from each well without disturbing the cells and the radioactivity level was determined in a Nuclear Chicago Gamma Counter. Base line release of ^{51}Cr was studied by incubating target cells in medium alone and was always less than 10% in a 4 hr assay and 20% in a 20 hr assay. Estimation of the maximum release of ^{51}Cr was determined by suspending 10^4 labelled target cells in 2 ml of water. After an incubation period of 4 or 20 hr at 37°, the radioactivity of 1 ml of supernatant was determined. Percentage specific lysis of target cells was calculated by the following formula. Percentage lysis = [(c.p.m. in supernatants of the test well − c.p.m. of base line release)/(c.p.m. released by water − c.p.m. of base line release)] × 100.

Lytic units

In some experiments, cytotoxicity levels in spleen cell preparations were quantified in terms of lytic untis (LU). For this purpose target lysis was studied at 100:1, 50:1, 25:1 and 12:1, E:T ratios. From the dose response curves, the E:T ratio (R) required to induce half of maximum lysis (ML, at E:T = 100/1) could be determined. Lytic units were calculated by the following formula: LU/10^6 spleen cells = [(50 × ML)/R]

NK, ADCC and R-ADCC

For estimation of ADCC activity, antibody-sensitized K562 target cells were used in the ^{51}Cr release assay. Sensitization was achieved either by adding a C57BL/6 anti-K562 antiserum directly to the assay medium or by pretreatment of K562 cells with 1:1000 dilution of the same antiserum. The optimal ADCC reaction was observed at 1:1000 dilution of anti-K562 antiserum added to the assay medium. R-ADCC activity was similarly determined either by adding an anti-H-2^b or anti-NK-1 antiserum to the assay

medium or using C57BL/6 spleen effector cells (H-2^b) pretreated with anti-H-2^b or anti-NK-1 antiserum (1/100 dilution). When anti-H-2^b antiserum was directly added to the ^{51}Cr release assay medium, optimal R-ADCC activity was obtained at a 1:100 to 1:200 dilution of the antiserum. Basal NK activity of mouse spleen cells could be determined in the ^{51}Cr release assay carried out with effector and target cells in the absence of antibody or before any antibody treatment.

RESULTS

Correlation between NK activity, ADCC and R-ADCC

To examine a possible relation between NK activity, ADCC and R-ADCC, we determined simultaneously the levels of these three types of cytotoxic activities in fifteen individual mouse spleen cell preparations. Data in Fig. 1a and 1b indicate that the basal NK activity correlated very well with the levels of ADCC ($r=0{\cdot}77$, $P<0{\cdot}007$) as well as R-ADCC ($r=0{\cdot}91$, $P<0{\cdot}0001$). Moreover, levels of ADCC and R-ADCC show a strong correlation between each other ($r=0{\cdot}84$, $P<0{\cdot}001$). High values for the correlation coefficients and the low P values for significance of the linear regression analysis shown in Fig. 1 make it likely that the three types of cytotoxic activities are related and may be mediated by the same lymphocyte subpopulation.

ADCC and R-ADCC are not additive phenomena

If the effector cells responsible for ADCC and R-ADCC are different, then the levels of target lysis induced by ADCC and R-ADCC effector cells should be additive. This possiblity was examined by determining the level of cytotoxicity in assays operating at the optimal levels for ADCC and then simultaneously inducing a R-ADCC reaction to see the effect on that level of activity. Since the lytic units (LU) constitute a quantifiable measure of cytotoxic cell activity, the results of cytotoxicity levels have been expressed in lytic units derived from the E:T ratio versus target lysis graphs. In addition, the assay duration was increased to 20 hr in order to increase the sensitivity of the assay (Saxena & Adler, 1979), and thereby obtain better dose–response curves. Results of two representative experiments are given in Fig. 2. In both experiments, the basal anti-K562 NK activity was significantly enhanced by the pretreatment of effector or target cells with R-ADCC and ADCC inducing antibody, respectively. When the antibody sensitized effector and target cells were simultaneously used, the resulting level of cytotoxic activity did not represent an additive R-ADCC+ADCC response. These results would be expected if the ADCC and R-ADCC reactions were mediated by a common population of effector cells.

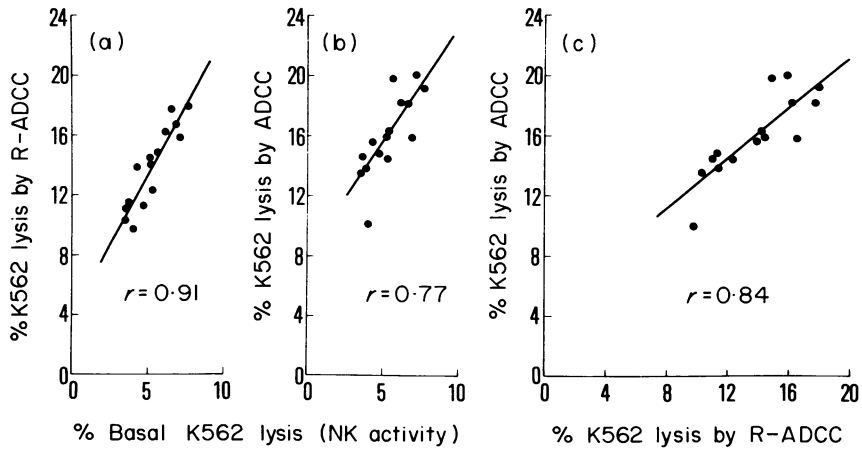

Figure 1. Correlation amongst basal NK, ADCC and R-ADCC levels in mouse spleen cell preparations. Fifteen individual C57BL/6 mouse spleen cell preparations were tested for anti-K562 cytotoxic activity in a 4 hr ^{51}Cr-release assay at E:T of 100:1. Cytotoxicity assay was carried out in the presence of C57BL/6 anti-K562 antiserum (1:1000 dilution) to determine ADCC levels, in the presence of DBA/2 anti C57BL/6 (anti-H-2^b) antiserum (1:200 dilution) to determine R-ADCC levels and in the absence of any antiserum to estimate basal NK activity levels. Each point in the graphs represent one spleen cell preparation. The linear regressions shown in (a), (b) and (c) were highly significant ($P<0{\cdot}001$ in each case).

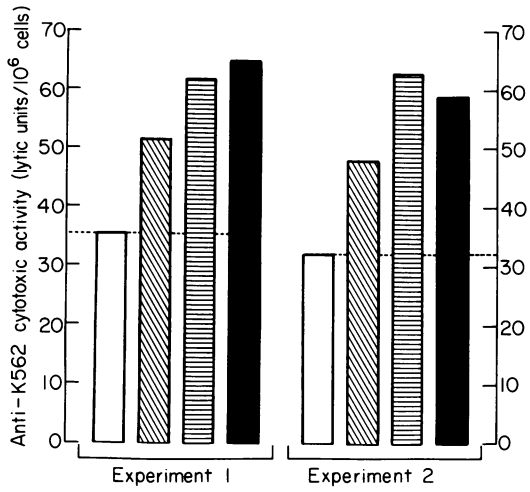

Figure 2. ADCC and R-ADCC reactions are not additive. K562 target lysis was determined in a 20 hr ^{51}Cr release assay at 100:1, 50:1, 25:1 and 12·1 E:T ratios. Lytic units/10^6 cells, as a quantifiable measure of cytotoxic activity, were calculated from the dose–response curves as described in Materials and Methods. R-ADCC reaction was induced by using anti-NK-1 antiserum treated effector cells and anti-K562 antiserum pretreated target cells were used to study the ADCC reaction. Method for antiserum pretreatmet of cells is given in legend to Fig. 3. R-ADCC + ADCC represents the simultaneous use of antibody-coated effector as well as target cells. (□) Basal NK; ▨ R-ADCC; ▤ ADCC; ■ R-ADCC + ADCC.

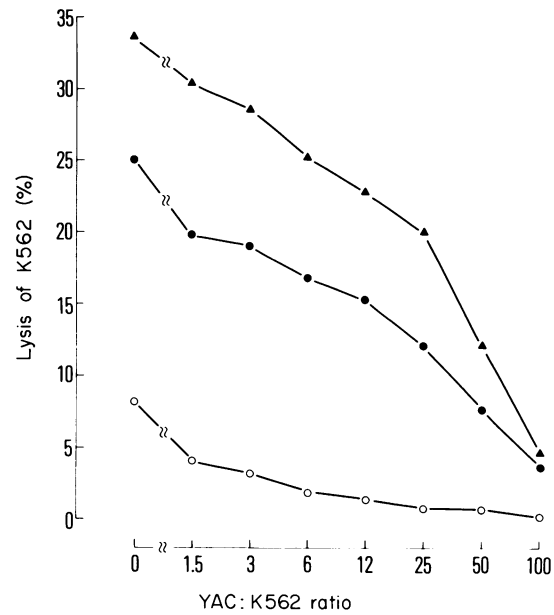

Figure 3. Inhibition of NK, ADCC and R-ADCC activities by YAC cells. Anti-K562 cytolytic activity of C57BL/6 mouse spleen cells was studied in a 4 hr ^{51}Cr-release assay at E:T of 100:1. In order to determine ADCC activity (▲—▲), K562 target cells were pretreated with a 1:1000 dilution of C56BL/6 anti-K562 antiserum and washed before use. R-ADCC activity (●—●) was assessed by using effector cells pretreated with 1:200 dilution of anti-H-2^b antiserum. Antiserum treatment was done by incubating the cells (10^6/ml target or 10^7/ml effector) with the antisera for 30 min at 4 followed by two washings. Control, untreated effector and target cells were used to estimate the basal anti-K562 NK activity (○—○). Different concentrations of YAC cells were present throughout the incubation. Each value of target lysis is a mean of three replicate observations.

Competitive inhibition of NK, ADCC and R-ADCC activities by YAC cells

YAC target cells are highly sensitive to NK-cell-mediated lysis and the addition of unlabelled YAC cells can inhibit the lysis of other NK sensitive ^{51}Cr-labelled target cells like K562 by competing for the interaction with NK effector cells (Saxena *et al.*, 1981b). If ADCC and R-ADCC are mediated by effector cells distinct from NK cells, it is conceivable that YAC cells would not compete for the lysis of K562 cells by ADCC or R-ADCC pathways and therefore would not inhibit ADCC or R-ADCC activities. Results in Fig. 3, however, indicate that the addition of YAC cells inhibited the lysis of K562 target cells by NK cells or by ADCC or R-ADCC pathways. The inhibition was dependent upon the concentration of YAC cells in the assay system, and was nearly complete at an effector: YAC ratio of 1:1. Addition of mouse thymocytes within these limits did not significantly inhibit any cytotoxic activity, indicating that the YAC-induced inhibition was specific in nature (results not shown). These results also suggest that a single population of effector cells may be responsible for NK, ADCC and R-ADCC activities.

DISCUSSION

After the recent demonstration of a new kind of antibody-dependent cytolytic activity (R-ADCC) in the non-T non-B compartment of mouse spleen lymphocytes (Saxena & Adler, 1979; Saxena *et al.*, 1980, 1981a, 1981b), it was of interest to determine if the R-ADCC activity is related to NK and/or ADCC cytolytic activities and whether the effector cell

mediating these different types of cytotoxic activities are identical or different. Our initial results indicated a strong correlation between the levels of NK, ADCC and R-ADCC activities in mouse spleen cells. A hypothesis indicating a common effector cell population for the three types of cytotoxic activities could easily explain these results. An important point regarding correlation experiments should be made here. Upon extrapolation, the linear regression lines in Fig. 1a and 1b would intersect at 4·1% lysis (R-ADCC) or 7·8% lysis (ADCC) values on the Y axis, corresponding to a zero basal NK activity. One interpretation of these results could be that R-ADCC and ADCC reactions can still take place in the absence of NK cells. This however may not be the case since we have found that if similar correlation lines are drawn between anti-YAC and anti-K562 NK activities, substantial anti-YAC NK activity can be shown by extrapolation, at zero anti-K562 NK activity (results not shown). Thus zero anti-K562 NK activity does not necessarily mean a lack of NK cells but only a lack of NK activity and may simply reflect the poor efficiency of K562 lysis by NK cells. It is interesting to speculate that ADCC and R-ADCC may represent an upgrading of a low efficiency NK cell–K562 type interaction towards a more efficient NK cell–YAC type interaction. Such a proposal would be supported by the recent observation by Brunda *et al.* (1981) indicating that the magnitude of the alloantibody-induced anti-K562 lytic activity (R-ADCC) paralleled the spontaneous anti-YAC NK activity in spleen cells. If ADCC and R-ADCC reactions are mediated by distinct and non-overlapping effector cell populations, it is conceivable that the simultaneous induction of these activities would result in an additive cytotoxicity response. Testing of this proposition however presented technical problems. Since H-2 antigens are expressed on all nucleated cells, treatment of spleen cells with anti-H-2 antiserum in order to induce R-ADCC reactivity, would also result in the generation of large concentrations of antibody-coated bystander cells which are known to interfere with the ADCC reaction (Halloran, Schirrmachar & Festenstein, 1974). In order to avoid the large concentration of antibody-coated bystander cells, we used an anti-NK-1 antiserum which reacts with $<5\%$ of the C57BL/6 spleen cells (Glimcher *et al.*, 1977) and has been shown to induce R-ADCC reactions (Saxena *et al.*, 1981b). Using anti-NK-1 antiserum to induce the R-ADCC reaction, we could demonstrate that the ADCC and R-ADCC cytotoxic responses were not additive, which is compatible with the idea of a common effector population.

A lack of additive effects favours a common effector hypothesis for ADCC and R-ADCC activities, but does not rule out the possibility that the effector cells responsible for both types of antibody-dependent cytotoxic activities could be distinct from NK cells. If ADCC/R-ADCC effector cells are indeed distinct from NK cells, the former should not by definition have any basal cytotoxic activity independent of antibody-induced activity. In such a situation, ADCC/R-ADCC effector cells would not require an antibody-independent recognition system to interact with target cells. However, YAC tumour cells which lack Fc receptors (Saxena *et al.*, 1981b), express target structures recognized by NK cells (Roder, Rosen, Fenyo & Troy, 1979), and are highly susceptible to lysis by NK cells, could markedly inhibit the NK, ADCC, as well as R-ADCC reactions indicating that besides the NK cells, ADCC and R-ADCC effector cells could also efficiently interact with YAC cells; a result which would be predicted by a common effector theory. Individuully, each of the arguments derived from the experiments indicating a common effector cell may not be fully convincing, but taken together the evidence appears to reinforce the argument suggesting that a common effector cell population may mediate NK, ADCC as well as R-ADCC reactions.

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