NATURAL CYTOTOXIC REACTIVITY OF MOUSE LYMPHOID CELLS AGAINST SYNGENEIC AND ALLOGENEIC TUMORS. II. CHARACTERIZATION OF EFFECCTOR CELLS

by

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Studies were performed to characterize the effector cells responsible for natural cytotoxicity of mouse lymphoid cells against a variety of syngeneic and allogeneic tumor lines. Since spleen cells from normal nude mice were found to be highly cytotoxic, they were used for most of these experiments. Only a small proportion of the reactivity was affected by treatment with anti-θ serum plus complement. Macrophages did not appear to be responsible for the reactivity, since treatment with carbonyl iron/magnet or carrageenan did not affect the levels of cytotoxicity. The effector cells were non-adherent, since passage over nylon columns resulted in a considerable increase in activity. The active cells did not have receptors for immunoglobulin or complement, since removal of cells with these receptors by columns or monolayers containing sheep erythrocyte-antibody (EA) complexes or EA-complement complexes did not remove activity. Antibody-dependent cell-mediated cytotoxicity appeared to be ruled out as the mechanism for natural cytotoxicity, since aggregated gamma globulin and a potent anti-immunoglobulin reagent did not inhibit reactivity, and since no role for humoral factors could be demonstrated. The natural effector cell was found to be quite labile at 37°C, losing much of its activity after 4 h. Since no surface markers could be detected on the effector cells, and the mechanism for cytotoxicity appeared distinct from others previously described, it is proposed that the natural cytotoxicity against mouse tumor cells is mediated by a unique subpopulation of lymphoid cells, which are tentatively designated N-cells.

In the accompanying paper (Herberman et al., 1975), we have described the natural cytotoxic reactivity of mouse lymphoid cells against a variety of syngeneic and allogeneic tumor lines. During that study, it was found that cells from athymic, nude mice had very high cytotoxic reactivity. Also, in a previous study, we found that the cytotoxicity of normal mouse lymphoid cells was not eliminated by treatment with anti-θ serum plus complement (Herberman et al., 1973). In contrast, cytotoxicity induced by inoculation of murine sarcoma virus (MSV) was abrogated by such treatment (Herberman et al., 1973). Since most direct cell-mediated cytotoxicity against alloantigens and tumor-associated antigens in the 51Cr release assay has been ascribed to, or was at least dependent on, T-cells (Cerottini and Brunner, 1974), it was of interest to perform detailed studies to determine the nature of the effector cells in this natural cytotoxicity system and to contrast them directly with MSV-immune cells, tested against the same target cells. It has

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been found so far (Cerottini and Brunner, 1974) that non-T-cell-mediated cytotoxicity is either due to antibody-dependent cell-mediated cytotoxicity (ADCC) or to macrophages (Evans and Alexander, 1971; Hibbs et al., 1972; Senik et al., 1974; Kirchner et al., 1975a, b). The present study, and a similar one by Kiessling et al. (1975) performed concurrently with ours, indicate that lymphocytes with no detectable cell surface markers are responsible for the natural cell-mediated reactivity.

MATERIAL AND METHODS

The mice, tumors and assay method were described in detail in the accompanying paper (Herberman et al., 1975).

Fractionation or pretreatment of effector cells

Treatment with anti-θ

AKR anti-θ C3H ascitic fluid was prepared by a method similar to that of Reif and Allen (1964). The procedure used has been described in detail elsewhere (Herberman et al., 1973). To demonstrate that the observed activity of the reagent was due to antibodies to θ-antigen and not to other lymphocyte alloantigens or other antigens, absorptions were performed with BALB/c brain homogenate, as previously described (Herberman et al., 1973). To demonstrate further the selective inactivation of T-cells with this reagent, C57Bl/6 spleen cells were treated with the anti-θ ascites plus complement and tested for stimulatory response to mitogens. The treated cells had no reactivity to phytohemagglutinin and concanavalin A (two T-cell mitogens) while they responded well to endotoxin, a B-cell mitogen (Kirchner and Holden, unpublished observations).

To determine the effect of anti-θ plus complement on cytotoxic reactivity, portions of 3×10^6 spleen cells were preincubated with a 1:2.7 final dilution of unabsorbed or absorbed ascites in RPMI 1640 medium with 10% fetal bovine for 30 min at room temperature. The cells were then washed once with RPMI 1640 medium, resuspended in a 1:3 dilution of non-toxic normal rabbit serum as the source of complement, and incubated for 45 min at 37°C. The surviving cells were then washed twice, counted and tested for residual activity in the cytotoxicity assay at a ratio of surviving attacker:target cells of 200:1.

Removal of adherent cells

Spleen cells from BALB/c nude mice were passed over nylon columns, prepared with teased, washed fibers from Fenwal Leukopaks (Fenwal Laboratories, Morton Grove, Ill., USA). The columns were first washed with Hanks’ balanced salt solution and then with RPMI 1640 medium with 0-20% fetal bovine serum. Cells at a concentration of 1×10^7/ml, suspended in RPMI 1640 medium plus 0-20% fetal bovine serum, were then placed on the columns and the flow rate adjusted to 50 drops/min. The columns were then washed again with 10 ml of medium and all of the eluted cells were pooled.

Removal of phagocytic cells with carbonyl iron and magnet

This procedure was performed as previously described in Kirchner et al. (1974).

Treatment of effector cells with carrageenan

This procedure was performed as previously described (Kirchner et al., 1975a, b), except that the spleen cells were incubated with 200-400 μg/ml for only 2 h at 37°C.

Treatment of effector cells with trypsin

Cells at a concentration of 1×10^7/ml were treated with 0.25% trypsin for 20 min at 37°C, and then washed twice. This procedure generally resulted in a loss of 20-30% of the cells.

Removal of cells with receptors for complement or immunoglobulins

In attempts to remove such cells, spleen-cell suspensions were passed over columns or incubated on monolayers containing sheep erythrocytes (E) and 7S anti-E antibodies (EA) (Cordis Laboratories, Miami, Florida, USA) or 19S anti-E antibodies (Cordis) plus complement (EAC). The EA and EAC were prepared as described by West and Herberman (1974). The E and EAC columns were prepared with Degalan beads (Golstein et al., 1972; Jondal et al., 1973). A small quantity of nylon fibers was placed in the bottom of a 12 ml plastic syringe and the Degalan beads were added to the 8 ml mark. The columns were washed with 15 ml of phosphate-buffered saline and then 4 ml of a solution of poly-L-lysine (Sigma Biochemical,
St. Louis, Mo., USA) at 25 μg/ml were poured through the columns, and another 4 ml were incubated on the columns for 20 min. The columns were again washed with 15 ml of phosphate-buffered saline, and then 5 ml of a suspension of E or EAC (at 1 x 10⁶/ml) in RPMI 1640 were incubated on the columns for 20 min. After washing again with 15 ml of phosphate-buffered saline, effector-cell suspensions (10⁵/ml at 37° C) were added to the columns and the effluent cells collected.

The E, EA and EAC monolayers were prepared as described by Kedar et al. (1974).

Treatment with anti-mouse γ-globulin

Various dilutions of goat anti-mouse γ-globulin reagent (kindly provided by Dr. R. Asofsky, National Institute of Allergy and Infectious Diseases) were added before mixture of attacker cells with labelled target cells. The procedure and reagent were the same as those previously described (Canty and Wunderlich, 1970; Herberman et al., 1973).

Treatment with aggregated human γ-globulin

Human γ-globulin (Miles Laboratory, Kankakee, Ill., USA) in Tris-buffered saline, pH 7.2, was heated at 60° C for 30 min, centrifuged at 10,000 x g for 15 min and various amounts of protein (determined by the Lowry method) from the pellet were added to the mixture of attacker cells and labelled target cells.

RESULTS

Effect of anti-θ treatment

Although the effect of treatment with anti-θ plus complement was previously studied (Herberman et al., 1973), this was done when tests were only made with normal C3H spleen cells against RBL-5 ascites target cells and low levels of lysis were seen. It was therefore important to perform further experiments with highly reactive spleen cells from nude mice, to confirm the previous results. Much of the natural cytotoxic reactivity remained after treatment of nude spleen cells with anti-θ plus complement (Table I). However, this treatment consistently caused some reduction in cytotoxicity. This effect appeared to be specifically related to reactions with θ-antigen, since brain-absorbed antiserum plus complement had no such effect.

<table>
<thead>
<tr>
<th>Pretreatment of spleen cells</th>
<th>BALB/c nude</th>
<th>MSV immune</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>38(0.9)</td>
<td>32(1.2)</td>
</tr>
<tr>
<td>Anti-θ + complement</td>
<td>25(0.7)</td>
<td>4(1.7)</td>
</tr>
<tr>
<td>Anti-θ absorbed with BALB/c brain + complement</td>
<td>37(1.9)</td>
<td>32(0.4)</td>
</tr>
<tr>
<td>Complement</td>
<td>35(0.6)</td>
<td>35(0.6)</td>
</tr>
<tr>
<td>Anti-θ</td>
<td>36(0.5)</td>
<td>31(0.7)</td>
</tr>
</tbody>
</table>

1 Percentage cytotoxicity (±st).

In addition, the other controls, including antibody alone and complement alone, had no effect on cytotoxicity. Furthermore, the anti-θ used in these experiments almost completely eliminated cytotoxic reactivity from spleen cells of mice immunized with MSV (Table I) and also eliminated stimulation of these cells by phytohemagglutinin.

Possible role of macrophages in natural cytotoxicity

Since macrophages have been shown to have cytotoxic effects on RBL-5 tumor cells and other tumor cells, in a growth inhibition assay (Kirchner et al., 1975a, b), it was important to determine their role in natural cytotoxicity. Three types of treatment, each of which removes or inactivates macrophages and eliminates the growth inhibitory effects, were used (Table II). Pretreatment of nude spleen cells with carbaryl iron and then removal of the phagocytic cells with a magnet had no effect on cytotoxic reactivity. Similarly, preincubation of the spleen cells with carrageenan had no effect when compared to the activity of cells incubated for the same period of time without carrageenan. Passage of the cells over a nylon column, in a media containing 20% FBS, resulted in a striking enhancement of activity. For comparison, the same procedures were also used with spleen cells from MSV-immune mice. As previously noted, these treatments had no effect on the MSV-induced reactivity (Kirchner et al., 1975b). However, it should be noted that passage of the
a series of experiments were performed to determine the possible role of this mechanism in natural cytotoxicity.

Pusanen and Asofsky (personal communication) have found that a potent goat anti-mouse γ-globulin reagent, G120, completely inhibits ADCC when added to the mixture of effector cells and target cells at dilutions of up to 1:1,000. After initial experiments in which high dilutions of this reagent had no effect in the natural cytotoxicity, a more extensive series of experiments, with a wide range of serum dilutions, were performed. Table V contains the data from a representative experiment. There was no appreciable inhibition of cytotoxicity at any concentration of antiserum. Similarly, this reagent had no effect on MSV immune reactivity.

Trypsin treatment of "armed" or antibody-producing effector cells has been found to inhibit ADCC (Schirrmacher et al., 1974). However, such treatment had no effect on natural cytotoxicity (Table II). Similarly, aggregated γ-globulin has been found to strongly inhibit ADCC (Larsson et al., 1973; MacLennan et al., 1973). When a wide range of concentrations of aggregated human γ-globulin was added to the incubation mixture, no effect on cytotoxicity was seen (Table VI).

If nude mice had high natural cytotoxic reactivity because of an ADCC mechanism, one might expect to find high titers of circulating natural antibodies which, when mixed with poorly reactive normal lymphoid cells, would cause strong cytotoxicity. Therefore, a wide range of dilutions of serum from nude mice were preincubated with normal BALB/c spleen cells and the cells were then tested for cytotoxicity against RBL-5 tissue culture target cells. Although the pooled spleen cells from the nude mice had 38% cytotoxicity, the sera from these mice did not cause increased cytotoxicity of the BALB/c cells (Table VII).

### TABLE VI

**EFFECT OF AGGREGATED HUMAN GAMMA GLOBULIN ON CYTOTOXIC REACTIVITY AGAINST RBL-5 TISSUE CULTURE CELLS**

<table>
<thead>
<tr>
<th>Amount of γ-globulin (mg)</th>
<th>Attacker cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BALB/c nude</td>
</tr>
<tr>
<td>None</td>
<td>37(0.3)</td>
</tr>
<tr>
<td>0.05</td>
<td>36(1.3)</td>
</tr>
<tr>
<td>0.5</td>
<td>37(1.0)</td>
</tr>
<tr>
<td>1.0</td>
<td>35(1.6)</td>
</tr>
<tr>
<td>10.0</td>
<td>35(1.3)</td>
</tr>
</tbody>
</table>

1 Heat aggregated γ-globulin added to incubation mixture of spleen cells and RBL-5 tissue culture cells.

### TABLE VII

**EFFECT OF SERUM FROM BALB/c NUDE MICE ON CELL-MEDIATED CYTOTOXICITY OF NORMAL BALB/c AGAINST RBL-5 TISSUE CULTURE CELLS**

<table>
<thead>
<tr>
<th>Serum dilution</th>
<th>Percentage cytotoxicity (±sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>6.8(0.5)</td>
</tr>
<tr>
<td>1:3</td>
<td>6.3(0.4)</td>
</tr>
<tr>
<td>1:10</td>
<td>6.1(0.4)</td>
</tr>
<tr>
<td>1:30</td>
<td>6.1(0.4)</td>
</tr>
<tr>
<td>1:100</td>
<td>6.9(0.4)</td>
</tr>
<tr>
<td>1:300</td>
<td>7.1(0.6)</td>
</tr>
<tr>
<td>1:1000</td>
<td>6.9(0.5)</td>
</tr>
</tbody>
</table>

1 0.1 ml serum dilution incubated for 45 min at 37°C in 0.1 ml containing 8 × 10⁵ normal BALB/c spleen cells. After washing, spleen cells were then mixed with ⁵¹Cr labelled RBL-5 tissue culture cells at a ratio of 200:1.

**Effect of preincubation of effector cells at 37°C**

During the course of experiments involving treatment of effector cells with materials at 37°C, it was noted that the untreated cells kept at the same temperature frequently had less reactivity than did another portion of the same cell suspension kept at 0°C. This indicated that the activity of natural effector cells might be labile at 37°C. Experiments were therefore...
inhibited (Table I). However, it is necessary to account for the partial reduction in the activity of nude spleen cells after treatment with anti-β plus complement. This effect appeared to be dependent on complement and on the presence of anti-β antibody, since absorption with β-antigen positive brain removed the inhibitory effect. Raff (1973) has reported that nude mice have small numbers of β-antigen positive cells. Although cells from nude mice do not give a detectable stimulation response to T-cell mitogens (Kirchner and Holden, unpublished observation), they may have some immature T-cells or precursors of T-cells which can contribute to the natural cytotoxicity. As a further difference of the natural reactivity from experimentally induced immune reactivity, we have found that inoculation of MSV into nude mice has not produced increased cytotoxicity (Holden and Herberman, unpublished observation). Other notable contrasts between the MSV immune cell reactivity and the natural reactivity include the presence of some natural reactivity in bone-marrow (Herberman et al., 1975), where we have never detected reactivity after MSV inoculation (unpublished observations), and the decline in natural reactivity after short periods at 37°C. The latency of natural cytotoxic reactivity at 37°C in vitro was a surprising finding which needs to be further studied. It is possible that the natural effector cells require continuous exposure to some factor present in vivo to retain activity at 37°C or that there is a rapid turnover of these cells in vivo. A more likely explanation is that the in vitro conditions at 37°C are deficient in some way and fail to maintain the functional integrity of this labile subpopulation of cells.

The effector cells in natural cytotoxicity do not have any of the properties of macrophages. They were not removed by treatment with carbonyl iron and magnet, and they were not inhibited by carrageenan, under conditions which markedly reduce the activity of macrophages in the growth inhibition assay (Kirchner et al., 1975a, b). In addition, the natural effector cells are not adherent to nylon columns. Such treatment resulted in quite significant enhancement in reactivity. The non-adherence of this cell population also provides some evidence against these cells being granulocytes or B-cells. B-cells have been found, by several investigators, to adhere to various fibers and surfaces (Julius et al., 1973; Wisloff and Folland, 1973). Kießling et al. (1975) also found that natural cytotoxicity was not affected by passage on anti-immunoglobulin columns, which more selectively remove B-cells and which have been shown to remove late phase, specific reactivity against MSV tumor lines in the microcytotoxicity assay (Lamon et al., 1973; Plata et al., 1974).

If the natural cytotoxicity cannot be directly related to the two previously described mechanisms for cytotoxicity against RBL-5 cells, i.e. to T-cell or macrophage cytotoxicity, and if it does not appear to be dependent on B-cells, the other known mechanism to consider carefully is antibody-dependent cell-mediated cytotoxicity. Although this form of cytotoxicity has not been studied with RBL-5 target cells, it has been observed with MSV-induced tumor cells in a long-term 51Cr release assay (Harada et al., 1971) and in visual microcytotoxicity assays (Pollack et al., 1972). However, the effector cells for natural cytotoxicity have several characteristics which differ from the cells mediating ADCC. The natural cytotoxicity was not affected by procedures which deplete cells binding to EA or to EAC. Addition of aggregated gamma globulin to the reaction mixture also had no effect. Therefore the natural cytotoxic cells appear to lack receptors for immunoglobulin or for complement, which have been described on the cells mediating ADCC (van Boxel et al., 1973; Kedar et al., 1974; Larsson et al., 1976; MacLennan et al., 1973; Wisloff et al., 1974). Mouse cells mediating ADCC have also been found to be adherent in most systems (Green et al., 1973b; van Boxel et al., 1973), which is in marked contrast to the natural cytotoxic cells. It might be argued that a different type of cell was responsible for this particular type of ADCC. However, procedures which have been shown to interfere with the ADCC mechanism had no effect on natural cytotoxicity. Addition of potent anti-γ globulin antibodies, known to inhibit ADCC (van Boxel et al., 1972; Henney et al., 1972) did not inhibit the reactivity. Since fresh culture grown target cells were used, the effector cells for ADCC would have to be “armed.” However, treatment of cells with trypsin, which has been shown to inhibit armed killer cells (Schirrmacher et al., 1974), had no inhibitory effect on natural cytotoxicity. Finally, addition of various dilutions of sera from nude mice.
which had high cell-mediated cytotoxic activity, did not enhance the reactivity of normal BALB/c spleen cells.

The characteristics of the effector-cell for natural cytotoxicity described here are very similar to those described by Kiessling et al. (1975). Greenberg and Playfair (1974) also described natural cytotoxicity of cells from NZB mice which were neither phagocytic nor of T-cell origin; however, they found a much more restricted organ distribution for the effector cells than we have seen (Herberman et al., 1975).

If the natural cytotoxic cells in the present study and in that of Kiessling et al. (1975) are not T-cells, B-cells or macrophages and are different from the effector cells for ADCC, what is the nature of these cells? No surface markers have been detected on these cells, with the possible exception, as noted above, of β-antigen on a portion of them. We were initially inclined to call the effector cells "null cells", since these cells had no detectable surface markers. However, this term has already been rather widely used to describe the cells which mediate ADCC, and these cells have receptors for immunoglobulin and/or for complement (Greenberg et al., 1973a; Kiessling et al., 1975). Therefore, to avoid confusion, we propose to tentatively call the natural cytotoxic cells "N-cells", pending identification of markers to place them in a definable subpopulation of lymphoid cells.

N-cell cytotoxicity represents a new mechanism for direct, specific cell-mediated cytotoxicity of tumor cells. This type of cytotoxicity needs to be seriously considered when cytotoxicity, even in immune systems, is found not to be due to T-cells or macrophages. One can no longer assume that all such effects are related to ADCC.

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REFERENCES


