Autoimmune Cytotoxic Granulocyte Antibodies in Normal Persons and Various Diseases

By S. Ian Drew and Paul I. Terasaki

Of 400 female and 58 normal nonimmunized male sera approximately 10% were cytotoxic for a panel of allogeneic granulocytes. Sera with strong alloreactivity were also autoreactive, which emphasized the large autoimmune component of most alloantisera against granulocytes. The cytotoxic granulocyte autoantibodies were complement dependent, of the IgM class, and exhibited optimum cytotoxic activity in vitro at 5°C precomplement incubation temperatures with papain-treated cells. The sera were unreactive with autologous or allogeneic B and T lymphocytes, monocytes, and red blood cells but were cytotoxic for adult and cord granulocytes, eosinophils, and chronic myeloid leukemia cells. Granulocyte autoantibodies were present in 53% of sera from 57 patients with systemic lupus erythematosus (p < 0.00002) but were not found in increased frequency in the sera of patients with 28 other diseases. We conclude that a single tissue-specific antigenic determinant called “G” may be present on granulocytes and is the target of naturally occurring autoantibodies.

Although HLA antigens are present on human granulocytes, there is evidence that granulocytes possess another set of alloantigenic specificities coded for by separate genes. Three neutrophil-specific genetic loci—NA, NB, and NC—have been defined by Lalezari and co-workers by use of leukoagglutination assays.1,2 We have attempted to look for granulocyte alloantigens by a complement-dependent cytotoxicity test.3,5 In the course of this work it was found that a large proportion of the cytotoxic reactions that had been observed was produced by autoantibodies to granulocytes. Thus many of the cytotoxic granulocyte antibodies were both allo- and autoreactive.

The present report investigates the serologic characteristics of granulocytotoxins and examines their relative frequency in the sera of normal persons and patients with various diseases.

MATERIALS AND METHODS

Cell preparation. Granulocytes were isolated from freshly drawn heparinized blood as previously described.5 An aliquot of each granulocyte preparation was papain treated (0.01%, papain, crude powder type I, Sigma Chemical) for 10 min at room temperature (23°C) and then washed three times in McCoy's medium containing 5%, fetal calf serum. Papain treatment of granulocytes under these suboptimal conditions for enzyme digestion is known to enhance the serologic reactivity of the cells without compromising cell viability or causing any apparent loss of cell surface antigen.5 Similar results have been observed with granulocytes pretreated with concanavalin A (results to be published). The granulocytes were adjusted to a concentration of 2.5 3 × 10⁶ cells/ml in Hank's balanced salt solution containing a mixture of ethyl and benzyl alcohol, 2 g/dl, to prevent the adherence of cells to the floor of the microtiter plate.
wells. We have found that alcohol alone does not have any effect on granulocyte viability or on antibody-mediated lysis of the cells. The percentage purity of the granulocyte preparations exceeded 90%, with less than 10%, contamination by lymphocytes and red blood cells. The viability of all cell preparations exceeded 95% under all test conditions in control wells with negative sera and complement alone (see below). The background of 5% dead cells did not increase with prolonged incubation times, with the use of the alcohol suspension medium, or by the use of papain-treated granulocytes.

**Assays.** The granulocyte cell suspension (1 μl) was added to 1 μl of a granulocyte antiserum and incubated for 30 min at 5°C, 22°C, or 37°C. Following this period, 5 μl rabbit serum, used as a source of complement, was added to the antibody-cell mixture and allowed to incubate at 22°C for 3 hr. The rabbit serum was preabsorbed with pooled human red blood cells to remove heterocytotoxins from the complement. The percentage of lysed cells stained with eosin dye was estimated by use of an inverted phase-contrast microscope. Leukoagglutination assays were carried out by incubation of nonpapain-treated granulocytes suspended in a 1% solution of EDTA, containing 0.2% azide and no alcohol, with granulocyte antiserum at 5°C or 37°C for 2-18 hr. In three instances, the cytotoxic reactivity of granulocytes not treated with papain was confirmed by incubating the cells with cytotoxic antibody and complement in the absence of alcohol and employing a fluorochromatic technique utilizing fluorescein diacetate and ethidium bromide for the detection of viable and killed cells, respectively.

**Antisera.** Sera reactive against granulocytes in the microcytotoxicity assay were identified by screening them against panels of random donor granulocytes. Only granulocyte antisera that were not cytotoxic for a panel of 70 peripheral blood lymphocytes that represented the majority of first and second locus HLA antigens were selected. Three normal male sera with no cytotoxic activity for a panel of more than 40 random granulocytes were used as negative control sera. No cytotoxicity was observed with even the strongest granulocyte antiserum if heat-inactivated rabbit complement (56°C for 30 min) was used. Likewise, no cytotoxicity occurred if granulocytes and (active) complement were incubated under optimum test conditions in the presence of negative control sera. These controls clearly define the complement-dependent and antibody-mediated characteristics of the assay in vitro. The cytotoxic endpoint of “strong” granulocyte antisera reacted against papain-treated cells at 5°C-22°C varied from 1:4 to 1:32.

F(ab′)2 fragments of a strongly cytotoxic granulocyte antiserum were produced by saturated (33%/w) ammonium sulfate precipitation and 24-h pepsin digestion (w/w) at pH 4.1 followed by dialysis against phosphate-buffered saline pH 7.2. Human IgG was aggregated by heating at 63°C for 1 hr and human IgM complexes prepared by incubating blood group substances obtained from the saliva of an antigen secretor with the respective isoantibody. Lymphocyte-dependent antibody (LDA) killing employed 2 x 10^5 pooled human effector lymphocytes and 5 x 10^5 human lymphocyte target cells coated with a 1:1000 dilution of a heterologous rabbit antilymphocyte antiserum.

**Absorptions.** Unless otherwise stated, absorptions were performed at a 3:1 serum to packed cell ratio. Cell-free supernatants were titrated against reference cells for their remaining antibody activity.

**Reproducibility.** The reproducibility of the cytotoxic reactions was examined by testing granulocytes obtained from five donors on two different bleeding dates against a panel of 108 granulocyte antisera. Paired cytotoxic reactions were compared for the same cell from the two different bleeding dates. Paired reactions were considered discrepant if the number of granulocytes killed by a given antiserum differed by more than 20% of dead cells. Based on 540 comparisons the calculated error rate for cytotoxic reactions was 4.5%.

**RESULTS**

Allogeneic reactivity. Over the last 2 yr more than 5000 sera from normal and alloimmunized donors were screened for the presence of granulocytotoxins and lymphocyte cytotoxins. Of these sera, more than 75% failed to show any cytotoxic reactivity (0% granulocytes killed) on repeated testing against random granulocyte panels. Owing to the presence of HLA on granulocytes, only granulocytotoxic antisera unreactive with lymphocytes were selected for further
tests. The lack of B lymphocyte antigens on mature granulocytes excluded cytotoxic reactivity because of B lymphocyte antibodies in the granulocyte antisera.

In a separate study of 400 normal female and 58 normal male sera, 42 (10.5%) of the female and 6 (10.4%) of the male sera were cytotoxic for more than 20% of a panel of 86 random allogeneic granulocytes. For the purposes of this study a granulocytotoxic antisera was arbitrarily defined as one cytotoxic for more than 20% of the panel of 86 donor granulocytes where the cytotoxic score against individual granulocyte preparations exceeded 50% of dead cells for a given serum. The pattern of serologic reactions produced by the cytotoxic granulocyte sera for allogeneic cells is shown in Fig. 1. The data were obtained using papain-treated granulocytes at a 5°C precomplement incubation temperature followed by a 3-hr postcomplement incubation period at 22°C. Positive cytotoxic reactions in which more than 50% of the granulocytes were killed by a given antiserum are denoted by the black squares. Cytotoxic reactions produced by the granulocyte antisera are arranged according to their percentage reactivity and the cells arranged according to their serologic reactivity with the panel of the sera.

Although the cytotoxic reactions given by a certain antiserum against allogeneic

![Fig. 1. Serograph of granulocytotoxic reactions. Black squares, positive cytotoxic reactions (more than 50% of cells killed) given by granulocyte antisera against papain-treated granulocytes at 5°C-22°C incubation temperatures.](image-url)
granulocytes appeared “specific” in that some granulocytes were killed and others were not, the reproducibility of the blank or “negative” serologic reactions was variable, and granulocytes isolated from “negative donors” on different bleeding dates usually reacted with the same cytotoxic antisera on subsequent testing. This occurred especially with the stronger antisera. From this it was supposed that some blank or “negative” reactions were attributable to weakly cytotoxic antisera or granulocytes that were resistant to lysis and that each cytotoxic antisera was potentially capable of recognizing antigens on all allogeneic granulocytes. Furthermore, the pattern of serologic reactions in Fig. 1 was suggestive of a single granulocyte antigen group on all granulocytes in that all the serologic reactions could be included within one hypothetical antigen group.

Evidence supporting the cytotoxicity data that granulocyte antisera were recognizing a single antigenic determinant(s) present even on “negative” cells was obtained from absorption studies. Each of three granulocyte preparations representing the least reactive cells against the panel of cytotoxic sera shown in Fig. 1, namely, cells 84, 85, and 86, were in turn used to absorb (2 × 10^8 cells/ml antiserum) three granulocytotoxic antisera. The sera selected were cytotoxic for 50%–80% of the panel of granulocytes but not cytotoxic for the three cell preparations used for the absorption study. In each case both the papain-treated and the untreated cell preparations completely absorbed the cytotoxic activity of the sera for a panel of 30 random granulocyte donors. The ability of “cytotoxic-negative” granulocytes to absorb the activity of granulocytotoxic antisera against all other granulocytes was a reproducible finding and supported the serologic evidence in Fig. 1, suggesting the presence of a shared antigenic determinant(s), termed “G,” on all granulocytes we have tested thus far.

**Autoreactivity.** Granulocytes isolated from ten normal persons (four males, six females; average age 38 yr) who had strong cytotoxic granulocyte antibodies against allogeneic cells were lysed in the presence of complement and the autologous antiserum. All the subjects with granulocyte autoantibodies were healthy and had no history of susceptibility to infection. None had received blood transfusions, and four of the six females had a history of previous pregnancies; none was on medication.

In two instances (M.H. and M.P.), multiple serum samples from the same donor were available over a 2-yr period. Representative and strongly granulocytotoxic sera from each subject showed normal immunoglobulin levels (IgG: 1290, 1155 mg/dl; IgA: 487, 230 mg/dl; IgM: 84, 80 mg/dl) and an absence of antinuclear, antithyroid, anti-smooth muscle, anti-parietal cell, and antiadrenal antibodies. Antimitochondrial and antirheumatoid factors were absent. Cryoglobulins were not detected. The cytotoxicity of the granulocyte antisera for the nonpapain-treated autologous granulocytes of M.H. is shown in Fig. 2. Overall optimum cytolysis of granulocytes occurred with 5°C incubation with antibody and 22°C incubation with complement compared with 22°C–22°C and 37°C–22°C. Similar results were obtained at 5°C–22°C if alcohol was omitted from the assay in vitro. No cell lysis was obtained with human complement or heat-inactivated rabbit complement. Total peripheral blood leukocyte counts in M.H. on three independent bleeding dates exceeded 6000 WBC/µl, with more than 50% segmented neutrophils and 2% eosinophils present on each occasion.
Fig. 2. Percentage cytotoxicity of granulocyte antibodies for autologous nonpapain-treated granulocytes at 5°C, 22°C, and 37°C precomplement incubation temperatures. Negative autoagglutination reactions at 5°C or 37°C shown.

A second male laboratory donor, M.P., had antibodies against 78% of a panel of allogeneic granulocytes. Six serum samples stored frozen over a 2-yr period were all cytotoxic for his autologous granulocytes (Fig. 3). As with M.H., the autocytophagocytotoxicity was temperature dependent, and optimum cytotoxicity occurred at 5°C-22°C. Papain treatment of the target cell increased the percentage of granulocytes killed by the panel of cytotoxic autoantibodies without compromising the cell viability. One of the six sera agglutinated the autologous granulocytes and only at 5°C (not shown).

Sera from four autoantibody producers, positive for 95%, 90%, 78%, and 65% of a random panel of allogeneic cells, were absorbed (2 x 10⁶ cells/ml antiserum) with autologous granulocytes. Following absorption at 5°C and 37°C, the reactivity of the granulocyte antisera for the autologous cell and a panel of ten random granulocytes was completely removed and no residual cytotoxicity for the target cells remained. Absorption of the granulocyte autoantiserum with a random granulocyte preparation was followed by a complete loss of autoreactivity. In two cases tested, 37°C heat eluates obtained from both papain-treated and nontreated granulocytes previously used to absorb a granulocytotoxic antiserum (positive for the absorbing cells and 95% of allogeneic granulocytes) were not cytotoxic for the same freshly isolated absorbing cells or allogeneic granulocytes.

Tissue specificity. A panel of 20 granulocyte sera cytotoxic for more than 80% of a random panel of granulocyte donors was selected. The sera were reacted against B and T lymphocytes purified from peripheral blood of 20 random persons. Although six sera had contaminating B lymphocyte antibodies, the remainder were specifically positive for granulocytes and failed to react with B or T lymphocytes. Negative cytotoxic results were obtained for three B and T lymphocyte preparations reacted against the autologous granulo-
Fig. 3. Percentage cytotoxicity of granulocyte antibodies for untreated and papain-treated autologous granulocytes at 5°C, 22°C, and 37°C precomplement incubation temperatures.

cytotoxic sera even if the lymphocytes were tested at 5°C or papain treated under the same conditions as granulocytes (Table 1). Pooled T lymphocytes or T cell lines (Molt-4, HSB-2) failed to absorb any cytotoxic activity of two granulocyte antisera tested, as did pooled chronic lymphocytic leukemia cells. Three monocyte preparations tested in parallel with granulocytes from the same donor did not react with the granulocyte antisera including the autologous cytotoxic sera, whereas random eosinophils obtained from three persons with allergic eosinophilia reacted with the majority of the panel of antisera. The

Table 1. Tissue Specificity of Granulocyte Cytotoxins: Percentage Positive Reactions Against 20 Granulocytoxic Antisera

<table>
<thead>
<tr>
<th></th>
<th>&gt;40%</th>
<th>0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult granulocytes*</td>
<td>70</td>
<td>B lymphocytes*</td>
</tr>
<tr>
<td>Cord granulocytes</td>
<td>3</td>
<td>T lymphocytes*</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>3</td>
<td>Monocytes*</td>
</tr>
<tr>
<td>CML blasts</td>
<td>1</td>
<td>Red blood cells*†</td>
</tr>
<tr>
<td>K562</td>
<td></td>
<td>Pooled platelets†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AML blasts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALL blasts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CLL blasts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CFU-C</td>
</tr>
</tbody>
</table>

Figures in parentheses represent number of cell preparations tested.
*Includes cytotoxicity or absorption with the autologous cell type.
†By absorption.
granulocyte antisera were cytotoxic for a chronic myeloid leukemia (CML) cell preparation that was comprised of 50% blasts and 22% myelocytes. K562, a CML cell line in culture that is known to lack HLA and B lymphocyte surface antigens, reacted with the granulocyte antisera as previously described. Kryopreserved acute myeloid leukemia (AML), acute lymphatic leukemia (ALL), and chronic lymphatic leukemia (CLL) blast cells tested were resistant to lysis. Interestingly, incubation of bone marrow cells from two normal donors with a strongly positive granulocytotoxic sera in the presence of rabbit complement failed to inhibit subsequent colony growth in agar relative to the appropriate controls. This preliminary finding suggests an absence of antigens defined by granulocytotoxic antisera on myeloid stem cells (CFU-C). Pooled platelets at a 1:1 ratio of packed cells to antiserum were unable to absorb the cytotoxic activity of seven granulocyte antisera tested. In regard to tissue specificity, the “G” antigen appears to be expressed primarily on more mature granulocytic forms and to be absent on lymphocytes, monocytes, and platelets.

Relation to red blood cell antigens. Evidence that anti-G is not related to red blood cell autoisoagglutinins was shown by absorption and direct screening tests. Autologous or pooled random red blood cells failed to absorb the activity of the cytotoxic autoantisera for granulocytes (Table I). Absorptions were carried out at 5°C with both papain-treated and untreated red blood cells at a 1:1 ratio of packed cells to antiserum. In addition, six strong granulocyte cytotoxic antisera reactive against the granulocytes of the serum producer were tested in triplicate at 4°C and at room temperature for red blood cell antibodies against nontreated and ficin-treated autologous, cord, and panel red blood cells. Hemagglutination tests were also performed using the indirect Coombs’ technique. No antibodies against I, H, P, M, or N red blood cell isoantigens were present in the granulocyte antisera. Three random cord granulocytes, presumed to lack the I antigen, reacted with the granulocyte antisera. More specifically, of a total of 45 granulocytotoxic antisera tested, more than 40% (19/45) were 80%-100% cytotoxic for cord granulocyte preparations. These data would exclude the possibility of anti-I as the factor responsible for the granulocyte auto-cytotoxicity.

IgM nature of antibodies. Gel filtration of the granulocyte autoantisera using a Sephadex 200 column separated a granulocytotoxic IgM fraction from an inactive IgG fraction. The purity of the individual IgM and IgG fractions was confirmed using goat anti-human anti-IgM and anti-IgG, respectively, in an Ouchterlony agar gel double diffusion assay. Furthermore, cytotoxic activity for autologous and allogeneic granulocytes could be abrogated by the addition of 2-mercaptoethanol (2-ME) to the antiserum (final serum concentration 1%-2% 2-ME). Granulocytes of one donor coated with F(ab’)2, fragments of a granulocytotoxic antiserum were not killed by the same whole antibody or a panel of granulocytotoxins tested. This would argue against the likelihood that immune complexes attached to granulocytes may bind complement and indirectly lyse the target cell as a “bystander” phenomenon. Furthermore, granulocytes coated with artificial human IgM or IgG immune complexes were not lysed in the presence of complement alone. Granulocytotoxins were not precipitated by less than 8% solutions of polyethylene glycol, nor did they block effector cell function in a standardized LDA assay.
Granulocytotoxins and disease. The frequency of granulocytotoxins in various diseases was examined by testing sera from patients at 5°C-22°C incubation temperatures against a panel of 40 papain-treated granulocytes obtained from random normal persons (Table 2). As before, a positive cytotoxic reaction was arbitrarily defined as one in which 50% or more of the granulocytes were killed by a given antiserum and a cytotoxic granulocyte antiserum as one which killed more than 20% of the panel cells. This allowed a comparison of the relative frequency of granulocytotoxins in the groups of subjects under study. As

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. of Patients Tested</th>
<th>Frequency of Positive Reactions (%)</th>
<th>Frequency Greater Than 20% (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Sera)</td>
<td>0% 1%-10% 10%-20% 20%-30% 50%-80% 80%-100%</td>
<td></td>
</tr>
<tr>
<td>SLE</td>
<td>57 17</td>
<td>8 2 16 5 9 52.6</td>
<td></td>
</tr>
<tr>
<td>Asthma</td>
<td>46 35</td>
<td>4 0 6 1 0 15.2</td>
<td></td>
</tr>
<tr>
<td>Bladder Ca</td>
<td>21 18</td>
<td>0 0 1 2 14.3</td>
<td></td>
</tr>
<tr>
<td>Aplastic anemia</td>
<td>94 57</td>
<td>21 4 9 1 2 12.8</td>
<td></td>
</tr>
<tr>
<td>Normal females</td>
<td>997 834</td>
<td>16 30 51 37 29 11.7</td>
<td></td>
</tr>
<tr>
<td>Normal males</td>
<td>108 94</td>
<td>1 2 7 2 2 10.2</td>
<td></td>
</tr>
<tr>
<td>Breast Ca</td>
<td>10 9</td>
<td>0 0 1 0 0 10</td>
<td></td>
</tr>
<tr>
<td>Gastric Ca</td>
<td>10 9</td>
<td>0 0 1 0 0 10</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>47 40</td>
<td>2 1 2 1 1 8.5</td>
<td></td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>24 21</td>
<td>1 0 2 0 0 8.3</td>
<td></td>
</tr>
<tr>
<td>Lymphoma</td>
<td>54 48</td>
<td>2 2 2 0 0 7.4</td>
<td></td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>71 61</td>
<td>4 1 4 1 0 7.0</td>
<td></td>
</tr>
<tr>
<td>Hashimoto disease</td>
<td>45 40</td>
<td>2 0 3 0 0 6.7</td>
<td></td>
</tr>
<tr>
<td>Sjögren syndrome</td>
<td>30 27</td>
<td>1 0 2 0 0 6.7</td>
<td></td>
</tr>
<tr>
<td>CLL</td>
<td>15 11</td>
<td>3 0 0 1 0 6.7</td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>46 42</td>
<td>0 1 2 1 0 6.5</td>
<td></td>
</tr>
<tr>
<td>Hodgkin disease</td>
<td>46 35</td>
<td>8 3 0 0 0 6.5</td>
<td></td>
</tr>
<tr>
<td>Hepatitis</td>
<td>50 45</td>
<td>1 1 1 1 1 6.0</td>
<td></td>
</tr>
<tr>
<td>Crohn disease</td>
<td>17 12</td>
<td>4 0 1 0 0 5.9</td>
<td></td>
</tr>
<tr>
<td>Graves disease</td>
<td>26 22</td>
<td>2 1 1 0 0 3.8</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>78 71</td>
<td>4 0 1 2 0 3.8</td>
<td></td>
</tr>
<tr>
<td>CML</td>
<td>31 29</td>
<td>1 0 0 1 0 3.2</td>
<td></td>
</tr>
<tr>
<td>Psoriasis</td>
<td>34 31</td>
<td>2 0 0 1 1 2.9</td>
<td></td>
</tr>
<tr>
<td>Ankylosing spondylysis</td>
<td>111 94</td>
<td>7 8 2 0 0 1.8</td>
<td></td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>54 53</td>
<td>1 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>Myasthenia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>myasthenia</td>
<td>7 7</td>
<td>0 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>Colon Ca</td>
<td>9 8</td>
<td>1 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>Cervix Ca</td>
<td>10 9</td>
<td>1 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>Lung Ca</td>
<td>10 9</td>
<td>0 0 0 0 0 0</td>
<td></td>
</tr>
</tbody>
</table>

Each serum was reacted against papain-treated granulocytes from a panel of 40 random persons at 5°C-22°C incubation temperatures. The number of sera that reacted at different frequencies against the panel of cells is given below, Ca, cancer.
controls, sera from 108 normal nonimmunized males and 997 parous and nonparous normal females were used. The number of patients in each disease category is given in Table 2 with each serum representing a single patient. Of the 29 disease states examined, the frequency of granulocytotoxins did not differ significantly from that of the normal controls in ankylosing spondylitis, aplastic anemia, asthma, Crohn disease, diabetes, Graves disease, Hashimoto disease, hepatitis, Hodgkin disease, leukemias, non-Hodgkin lymphoma, multiple sclerosis, myasthenia gravis, psoriasis, rheumatoid arthritis, sarcoidosis, and Sjögren syndrome. It is interesting that in most instances the reactivity of sera from patients with disease was less than the reactivity of sera from normal persons. This may either be produced by sampling differences or possibly may reflect variations in sera storage times.

In contrast to the small variations noted in the 28 other diseases, systemic lupus erythematosus (SLE) sera showed a marked increase in the frequency of granulocyte cytotoxins, and as many as 53% of sera from 57 patients with SLE reacted with the granulocyte panel. Although antibodies to random T and B enriched populations were present in some SLE antisera, 43% (13/30) of the granulocytotoxic sera were positive for granulocytes and negative for T lymphocytes, compared to 10% of sera in the normal male population ($\chi^2 = 15.7, p < 0.002$). Column fractionation of an SLE antiserum showed granulocytotoxic activity present only in the IgM immunoglobulin fraction.

**DISCUSSION**

The introduction of low precomplement incubation temperatures together with papain-treated granulocytes and prolonged incubation times has enhanced the serologic reactivity of the granulocyte target cell in vitro. In addition, cold incubation temperatures have favored the expression of autoimmune granulocyte antibodies. That the enhanced reactivity represents an artifact of the system in vitro is unlikely for the following reasons: (1) the cytotoxic reactions are both complement dependent and mediated by IgM antibody; (2) background lysis of cells under all conditions in vitro remained at less than 5% dead cells; (3) lysis of granulocytes by autologous sera can be shown with nonpapain-treated granulocytes (Figs. 2 and 3) and in the absence of alcohol; and (4) over 75% of randomly selected sera showed no evidence of cytotoxicity.

By optimizing the assay in vitro, granulocyte antibodies previously thought to define group-specific antigens under suboptimal conditions of temperature and target cell reactivity now appear to lack specificity and to define instead a single antigenic determinant present on all granulocytes (Fig. 1). Evidence for a common shared antigen on granulocytes is the ability of randomly selected granulocytes to absorb the cytotoxic activity of granulocyte antisera against all other granulocyte donors tested. To date we have been unable to find an exception to this observation.

Granulocyte autoantibodies are best demonstrated by cold-temperature incubation, although high-titer autoantibodies may be cytotoxic at 22°C and 37°C. As such, reactivity at 37°C would not distinguish between reactions due to autoantibodies versus granulocyte alloantibodies. However, this does not negate the likelihood that true alloimmune antibodies cytotoxic for granulo-
cytes do exist especially in sera obtained from heavily immunized (i.e., multi-
transfused) patients. Such antibodies, by definition, should not react with
autologous granulocytes, nor should absorption with autologous cells remove
the cytotoxic reactivity against allogeneic granulocytes. Our present findings
serve to emphasize the importance of suspecting sera with alloreactivity of
being autoreactive. This was evidenced by the loss of alloreactivity of four
strongly cytotoxic antibodies following their absorption with autologous
granulocytes.

Autoantibodies to neutrophils have been detected by opsonization and
leukoagglutination tests. In the present study the lack of autoagglutination by
the autologous cytotoxic antisera distinguished the two types of antibodies.
However, in separate studies we have found that approximately 40% of granulo-
cytic antisera may agglutinate granulocytes in the cold (results to be pub-
lished). In this regard, autoagglutinating and 2-ME-sensitive neutrophil-specific
antibodies reactive at cold (4°C) temperatures have been described and may
be analogous to the granulocytotoxins described here.

Our results indicate that the target antigen against which granulocyte auto-
antibodies are directed has tissue specificity and differentiates certain cells of
myeloid lineage from other peripheral blood elements. The antigen is present on
both mature adult and cord granulocytes, eosinophils, one chronic myeloid
blast cell population tested, and a cultured CML cell line (K562). On the other
hand, it is absent from T and B lymphocytes, monocytes, red blood cells, AML,
ALL, and CLL blast cells, platelets, and probably myeloid stem cells (CFU-C).
From this, the autoantigen may be considered granulocyte-specific, and we pro-
pose the designation G for this antigen. The presence of G on all granulocytes
would exclude any association between G and antigen systems such as ABO
and HLA.

Although antibodies to autologous granulocytes are compatible with normal
peripheral blood leukocyte counts and good health, the same granulocytotoxic
autoantibodies as described here have been associated with neutropenia in pa-
patients with levamisole-induced neutropenia (two cases; results to be published),
Felty-like syndrome (one case), and in seven patients with “autoimmune
neutropenia” studied to date (unpublished). Whether the granulocytotoxic anti-
body is causally related to the neutropenia or occur as a concomitant
epiphemomenon remains to be established.

Investigation of 29 different diseases for the relative frequency of granulocyte
cytotoxins showed that in 28 of the diseases studied granulocyte antibodies are
unlikely to play a significant role. However, in SLE a marked increase in auto-
antibodies to granulocytes was found. Granulocytes should therefore be added
to the long list of autologous tissues against which an SLE patient produces
antibodies. As such, granulocytotoxic antibodies may possibly be implicated
in the granulocytopenia that sometimes accompanies this disorder.

Serologic models analogous to the G-antigen system exist. First and most
likely is that the restricted cellular reactivity of granulocyte autoantibodies
suggests that they may be grouped together with other known tissue-specific
autoantibodies such as autoantibodies directed against thyroid tissue, adrenal
tissue, smooth muscle, gastric mucosa, etc. However, granulocyte autoanti-

bodies. In addition, the latter type of antibodies was not detectable in granulocytotoxic antisera examined from two normal subjects, nor was the incidence of granulocytotoxins increased in autoimmune disorders other than SLE. The presence of a tissue-specific antigen on granulocytes would support serologic evidence for a single shared antigenic determinant(s) on all granulocytes. Second, our data do not exclude the possibility that granulocyte autoantibodies are reacting against a ubiquitous bacterial or viral antigen(s) present on the cell surface of mature granulocytes. Third, certain analogies exist between autoanti-I red blood cell antibodies and antibodies to the G antigen on granulocytes.23,24 Both antibody types (1) are cytotoxic for autologous as well as allogeneic cells, (2) define antigenic determinant(s) present on cells from almost all persons, (3) are present in normal nonimmunized persons, (4) are optimally detected at low incubation temperatures, (5) are IgM in nature, (6) are most reactive with enzyme-treated target cells, and (7) are associated with some “autoimmune” cytopenic states. However, granulocytotoxins differ from autoanti-I antibodies in that (1) cord granulocytes react similarly to adult granulocytes, (2) autologous red blood cells do not absorb out the G activity, and (3) the strongest granulocyte cytotoxic autoantisera had no demonstrable anti-I activity against adult red blood cells.

The presence of granulocyte autoantibodies in normal individuals raises the question of existing homeostatic and biologic mechanisms that enable granulocytes to escape the sensitization by autoantibody in vivo. Both target cell lysis and the absorption of granulocyte autoantibodies at physiologic temperatures have demonstrated the ability of the antibodies to bind to granulocytes at 37°C. This was unexpected in view of the optimum cytotoxicity demonstrated at cold incubation temperatures. Reasons for these observations are not clear but raise the following interesting possibilities: (1) Granulocytes may be able to escape immune sensitization at physiologic temperatures before cell destruction ensues. At a cellular level, the rapidity with which granulocytes cap and endocytose bound antibody has been demonstrated.25 In addition, granulocytes may internalize or shed antibody-antigen complexes or inactivate surface-bound antibody by cellular enzymes, thus permitting the cell to resist autoimmune lysis. Cold incubation temperatures would serve to “freeze” the cell membrane and inhibit metabolic functions, allowing the removal of surface-bound antibody. Indeed, the cold dependence of the assay in vitro may promote cytotoxicity at the cellular level rather than influence the affinity of antibody binding which appears to occur independent of incubation temperature. As such, granulocyte autoantibodies would differ from cold hemolysins, which lose affinity with increasing temperature. (2) Events at a cellular level, similar to those proposed above, could explain the ability of the same granulocyte antibody to be both innocuous (in normal subjects) and pathologic (in patients with SLE). Here, defects of the target cell (for example, secondary to viral infection) could inhibit biologic escape mechanisms and thus select for neutropenia in those individuals with granulocytotoxins.

Finally, the inability of human complement to mediate lysis of granulocytes sensitized with autoantibody in vitro favors alternate methods of granulocyte elimination and destruction in vivo. One such possibility is that sensitized granulocytes may bind complement that remains at the cell surface after the
antibody has been removed. Complement-coated granulocytes may be more susceptible to damage by the reticuloendothelial system. Similar mechanisms of cell elimination have been proposed for the action of red blood cell autoantibodies. As such, the cytotoxicity in vitro of granulocytes may not necessarily imply lysis in vivo of these cells.

ACKNOWLEDGMENT

We wish to thank Brenda Carter, Dan Guidera, and Kathy Lee for the excellent technical assistance. We thank Dr. Domenico Bernoco, Dr. Ronald Billing, and Dr. Martin Cline for their constructive suggestions and help with this study.

REFERENCES

Autoimmune cytotoxic granulocyte antibodies in normal persons and various diseases

SI Drew and PI Terasaki