Toxicity of Anti-dog Lymphocyte Serum to Normal and Leukemic Human Lymphocytes

By Robert Schrek, Frederick W. Preston and Albert A. Dietz

A PRELIMINARY REPORT showed that rabbit anti-dog lymphocyte serum was more toxic in vitro to leukemic than to normal human lymphocytes. This paper presents detailed data on the toxicity of this immune heterologous serum. As the dog lymphocytes have the Forssman antigen and rabbits are Forssman negative, it was necessary to consider whether the observed toxic reaction of the immune serum was due to Forssman antibody. To study this question, other immune heterologous sera were prepared and tested for their toxicity to leukemic and normal human lymphocytes.

MATERIALS AND METHODS

Thoracic duct lymph of dogs was obtained by catherization of the thoracic duct. The cells were washed with Hank's tissue culture fluid and suspended in the fluid to give 10^8–10^9 viable lymphocytes per milliliter. The viable cell count was made by the eosin dye method. Rabbits were injected subcutaneously with 2–10 × 10^7 dog lymphocytes plus complete Freund’s adjuvant. Four weeks later the rabbits were given 3 daily intravenous injections. The rabbits were bled 4 days after the last injection. Anti-lymphocyte sera from rabbits, rats, and guinea pigs were also obtained following 4 weekly subcutaneous injections of suspensions of 10^8–10^9 lymphocytes from lymph nodes of dogs.

To prepare rabbit globulin, the proteins of the immune serum were precipitated at and washed with 40 per cent saturated ammonium sulfate and the precipitate was dialyzed against distilled water. The proteins precipitating on dialysis were solubilized with phosphate buffer of pH 6.7. The solution was passed through a DEAE-cellulose column equilibrated with 0.01 M phosphate buffer pH 6.7, and the protein eluted with this buffer was dialyzed and lyophilized. The DEAE-cellulose fraction was 3 per cent of the original amount of protein; disc electrophoresis showed it to be essentially pure γ-globulin. Fractionation of human serum by this method has been shown to yield pure γ-immunoglobulin.

Heparinized blood was obtained from persons with normal hemograms and from patients with chronic lymphocytic leukemia. The erythrocytes were allowed to sediment at 37 C. for
1 to 2 hours. The cells in the supernatant plasma were washed. Purified lymphocyte suspensions were obtained as described previously by allowing the adherence of granulocytes and monocytes to glass. The lymphocytes were suspended in 10 per cent heat inactivated (56 C. 30 minutes) normal human serum in Fischer's medium No. 147G (Grand Island Biological Co., Grand Island, N. Y.). The final concentration was approximately 500,000 lymphocytes per milliliter.

To test the cytotoxicity of an immune serum, 0.1 ml. of heat inactivated serum was added to 0.5 ml. of lymphocyte suspension and then 0.4 ml. of fresh rabbit serum was added as complement. The controls included cells plus heat inactivated normal serum and complement. The mixtures in small test tubes were incubated for 1 hour at 37 C. and were then transferred to special slide chambers. The cells were examined and viable lymphocytes were counted with an inverted phase contrast microscope. A final count of viable lymphocytes was made 20 hours after incubation of the slide chambers. The number of viable lymphocytes surviving in the slide chambers was expressed as a percentage of the number of viable lymphocytes in the control untreated suspension before incubation.

**RESULTS**

**Immune Rabbit Serum**

Viable cell counts were made by phase contrast microscopy of lymphocytes incubated for 20 hours in a media containing 10 per cent or less of heat inactivated anti-dog lymphocyte serum from rabbits plus 40 per cent fresh normal rabbit serum as complement. Figure 1 shows the effect of dosage of the immune serum on the survival of lymphocytes from 11 patients with chronic lymphocytic leukemia and from 18 normal persons. The complement itself, fresh rabbit serum, was slightly more toxic to the leukemic than to normal lymphocytes (56.5 and 77.7 per cent survival, respectively). The data on 1

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**Fig. 1.—Effect of rabbit anti-dog lymphocyte serum on the survival of human lymphocytes from 11 patients with chronic lymphocytic leukemia and from 18 persons with normal hemograms.** The abscissae show the dilution of the immune sera on a logarithmic scale; the ordinates, the percentage of lymphocytes surviving 20 hours on a probit scale.
TOXICITY OF ANTI-DOG LYMPHOCYTE SERUM

Fig. 2.—The distribution of titers of immune sera (rabbit anti-dog lymphocyte) for lymphocytes from 11 leukemic patients and 18 normal persons.

patient were not included because his blood lymphocytes were very sensitive to the normal rabbit serum (only 15 per cent survival in 20 hours). On the average, the immune rabbit serum plus rabbit complement were more toxic to the leukemic than to normal lymphocytes.

The titer of the serum was defined as the concentration of immune serum that allowed the survival of only 10 per cent of the lymphocytes. It is seen in Figure 1 that the average titer was approximately 1:100 for leukemic lymphocytes and was more than 1:10 for normal lymphocytes. In individual experiments the titers were calculated by interpolation. The titer was greater than 1:10 in cells from 13 out of 18 normal persons and was 1:100 or less in only 1 case (Fig. 2). In contrast, the lymphocytes from 7 of 11 patients with chronic lymphocytic leukemia gave titers of 1:100 or less. The difference in titers for normal and leukemic lymphocytes is statistically significant (p < .01).

A summary of clinical data on the 11 leukemic patients is given in Table 1. The white blood cell counts varied considerably. No significant correlation was obtained between absolute lymphocyte count and sensitivity of the lymphocytes to the immune serum.

The effect of the rabbit anti-dog lymphocyte serum on lymphocytes from 3 dog lymph nodes was determined in a similar manner except that 10 instead of 40 per cent rabbit serum was used as complement. The titer of the serum was 1:1000 against dog lymphocytes as compared to 1:100 against human leukemic lymphocytes.

The data were analyzed to determine whether the sensitivity of leukemic lymphocytes might be due to the heterophilic antigen in blood group A cells. Blood group O erythrocytes occurred in 5 of 11 leukemic patients and in 9 of 18
normal persons. The median titers of the immune sera were 1:110 for the leukemic and > 1:10 for the normal lymphocytes. No correlation was found between the sensitivity of normal and leukemic lymphocytes and the blood group.

Purified γ globulin prepared from immune rabbit serum plus fresh rabbit serum was found to be significantly more toxic to leukemic than to normal lymphocytes (Table 2).

Other Heterologous Sera

Sera or globulin were obtained from rats, guinea pigs, and horses immunized with dog lymphocytes. The rats are Forssman negative and guinea pigs and horses, Forssman positive.2 The immune rat serum plus rabbit complement were toxic to leukemic but not to normal human lymphocytes (Table 2). In contrast, heat inactivated immune guinea pig serum and horse globulin plus complement were toxic both to normal and leukemic lymphocytes with little or no difference in sensitivity. Only the rat and the rabbit yielded immune sera which differentiated between normal and leukemic lymphocytes.

Rabbit anti-sheep erythrocyte serum (Forssman antiserum) in a 1:10 dilution plus rabbit complement were not appreciably toxic to normal human lymphocytes or to lymphocytes from 4 patients with chronic lymphocytic leukemia.

Cytology

Cytologic observations of the effect of the various reagents were made on the lymphocytes in slide chambers by phase contrast microscopy. Control suspensions without immune serum or complement showed, both before and after 20 hours of incubation, viable lymphocytes (Fig. 3) with clearly visualized nuclear and cytoplasmic structures and with cytologic characteristics of viable normal and leukemic lymphocytes described previously.5 The heat inactivated immune serum without complement caused the formation of small and large masses of viable lymphocytes.
Table 2.—In Vitro Effect of Anti-Dog Lymphocyte Serum and Globulin from Different Species on Normal and Leukemic Human Lymphocytes

<table>
<thead>
<tr>
<th></th>
<th>Rabbit*</th>
<th>Species immunized with dog lymph nodes</th>
<th>Rat</th>
<th>Guinea Pig</th>
<th>Horseb</th>
<th>Normal lymphocytes</th>
<th>Chronic lymphocytic leukemia</th>
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<tr>
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<td>5</td>
<td>8</td>
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<td>Dilution of immune serum or globulin</td>
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<tr>
<td>1:10</td>
<td>18.0</td>
<td>67.4</td>
<td>0.6</td>
<td>45.1</td>
<td>0</td>
<td>0</td>
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<tr>
<td>1.30</td>
<td>4.7</td>
<td>58.7</td>
<td>2.9</td>
<td>5.0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>1:100</td>
<td>20.6</td>
<td>69.9</td>
<td>34.6</td>
<td>69.9</td>
<td>6.6</td>
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<tr>
<td>1:300</td>
<td>42.6</td>
<td>63.7</td>
<td>76.3</td>
<td>1.1</td>
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<tr>
<td>1:1000</td>
<td>77.8</td>
<td>98.7</td>
<td>44.2</td>
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<td>87.9</td>
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<td>58.3</td>
<td>83.5</td>
<td>66.9</td>
<td>92.6</td>
<td>49.7</td>
</tr>
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* Globulin.
* Globulin contributed by Dr. T. E. Starzl.
* LL=lymphocytes from patients with chronic lymphocytic leukemia.
* NL=normal lymphocytes.
* Control = normal human serum.
* Complement = fresh rabbit serum, 40 per cent.
* Significantly different from the percentage survival for normal lymphocytes (< 0.01) according to the Wilcoxon Rank Sum test.15

Heat inactivated rabbit anti-dog lymphocyte serum plus rabbit complement caused degenerative changes in leukemic lymphocytes during incubation at 37 C. for 1 hour. The most common early degenerative changes affected primarily the nuclei of the leukemic cells. The nuclei usually rounded up and appeared more contrasty and had a small amount of clear nucleoplasm and dark nucleoli and marginated chromatin masses (Fig. 4). This early degenerative change was seen even in a few nuclei which still had an indentation of the nuclear wall (Fig. 5). The degenerating cells themselves, also usually rounded up and had only a slight or moderate amount of cytoplasm. The cell walls were not visible. In many of these cells, one pole of the nucleus had a small or large, crescentic, clear paranuclear vacuole (Fig. 6). Paranuclear vacuoles were also seen in a few cells which showed no other cytologic abnormality (Fig. 7). On further incubation, the nuclear structures became blurred and finally the nuclei became homogeneous, moderately gray, and pyknotic. The paranuclear vacuoles frequently persisted in dead cells with pyknotic nuclei (Fig. 8).

DISCUSSION

Rabbit and rat anti-dog lymphocyte sera plus rabbit complement were found to be more toxic to leukemic than to normal human lymphocytes. This finding raises the question whether leukemic lymphocytes contain an antigen which is not present or evident in normal lymphocytes and which is, in some way, related to an antigen in dog lymphocytes. Specific nonviral antigens in lymphomas of mice,6 rats,7 and hamsters8 have been reported. In addition, Klein
Figs. 3–8.—Photomicrographs by phase contrast microscopy of unstained cells, 2000×.

Fig. 3.—Lymphocytes from a patient with chronic lymphocytic leukemia. The cells have been incubated with normal fresh rabbit serum for 20 hours. All the cells are viable as indicated by the distinct nuclear structures including dark margined chromatin masses, nucleoli and indentation of the nuclear wall.

Figs. 4–7.—Early changes in leukemic lymphocytes incubated for 1 to 2 hours with inactivated rabbit anti-dog lymphocyte serum and rabbit complement. The lymphocytes in Figures 4, 5, and 6 have very clear nucleoplasm with persistence of nuclear indentation (Fig. 5) and of margined chromatin masses. The cells in Figures 6 and 7 have a crescentic paranuclear vacuole associated with clear nucleoplasm in Figure 6 and with no apparent nuclear change in Figure 7. The cytoplasm of the cells show little or no change.

Fig. 8.—Late changes in a leukemic lymphocyte incubated for 20 hours with inactivated rabbit anti-dog lymphocyte serum and rabbit complement. The cell has a homogeneous, dark gray, pyknotic nucleus and a paranuclear vacuole.
et al. obtained by immunofluorescent staining evidence of a specific antigen in Burkitt’s lymphoma cells and of an antibody in the sera of patients in remission from Burkitt’s lymphoma. These findings support the hypothesis that lymphoma and leukemic cells may have specific antigens.

In a previous study, normal rabbit sera were found to be more toxic to leukemic than to normal human lymphocytes. The reaction, however, varied considerably with sera from different rabbits. Similarly, Schlesinger and Amos reported that normal rabbit sera were toxic to lymphocytes from some patients with chronic lymphocytic leukemia. They attributed the toxicity to a natural heteroantibody which required complement to produce a cytotoxic effect on leukemic lymphocytes. Both the natural and the induced (anti-dog lymphocyte) antibody of rabbits were more toxic to leukemic than to normal lymphocytes.

Previous work has presented evidence that human leukemic lymphocytes may be derived from one type of lymphocyte called the “T” lymphocyte which is apparently present in greater numbers in rat than in human blood. This hypothesis was developed to explain the finding that many of the in vitro reactions of human leukemic lymphocytes were similar to those of normal rat lymphocytes. We, therefore, have to consider whether the hypothesized heterophilic antigen in leukemic lymphocytes is also present in the “T” type of normal human lymphocyte. Old et al. have reported a specific “TL” antigen in mouse lymphomas which is also present in thymic cells of some mouse strains. Paranuclear vacuoles were seen in the present study in many leukemic lymphocytes treated with the rabbit anti-dog lymphocyte serum. Paranuclear vacuoles were also seen by Schlesinger in a high percentage of murine thymic cells but in relatively few lymph node cells which were undergoing cytolysis following isoantibodies or heterologous sera. Sachs and Fogel reported the presence of Forssman and RAM antigens in renal tumors induced by virus in hamsters and rats, respectively. These antigens were also present in cultures of embryonic tissues of these species. In addition, a heterophilic antigen has been reported in normal human leukocytes by Rapaport who found that persons immunized with homologous leukocytes or skin grafts developed hemagglutinins against rat, sheep, and guinea pig cells. His work suggests that there is a common or related heterophilic antigen in human leukocytes and rat erythrocytes. There is, however, no experimental data to correlate Rapaport’s finding of a heterophilic antigen in normal leukocytes and the present presumptive finding of a heterophilic antigen in leukemic lymphocytes. An analysis of the data in this study and in the literature suggests that human leukemic lymphocytes contain an antigen which may be present in some normal human leukocytes and which is related to an antigen in normal dog lymphocytes but which is not the classic Forssman antigen.

Observations were made of the cytology of cells treated with rabbit anti-dog lymphocyte serum plus rabbit complement in an attempt to determine the mechanism and site of the cytologic action. The first degenerative changes appeared to be in the nucleus. In addition, crescentic paranuclear vacuoles were seen in many dying cells and in some cells these vacuoles appeared to be
the first cytologic degenerative change. In contrast, other workers have reported that cells treated with specific heterologous and homologous antisera developed cytoplasmic edema and peripheral bullae. The early nuclear changes observed in leukemic lymphocytes suggested that the nucleus or nuclear membrane is the primary site of action of the rabbit anti-dog lymphocyte serum plus complement. It is possible, however, that the nuclear changes are secondary to injury of the cellular membrane.

**Summary**

Rabbit anti-dog lymphocyte serum plus rabbit complement had a rapid cytocidal effect on blood lymphocytes from 11 patients with chronic lymphocytic leukemia. The titer of the immune sera varied from 1:42 to 1:530 with a mean titer of 1:100. The sera showed minimal or no toxicity to lymphocytes from normal persons with a titer of > 1:10 in 13 of 18 cases. A similar differential toxicity to normal and leukemic lymphocytes was observed with rat anti-dog lymphocyte serum. However, guinea pig and horse anti-dog lymphocyte sera or globulin were equally toxic to normal and leukemic human lymphocytes. Leukemic lymphocytes incubated with rabbit anti-dog lymphocyte serum and rabbit complement showed early nuclear degenerative changes and paranuclear vacuoles. The cytologic degenerative changes suggested that the nucleus or nuclear membrane may be the first site of action of the immune serum.

**SUMMARIO IN INTERLINGUA**

Sero de conilio anti lymphocytos canin insimul con complemento de conilio habeva un rapide effecto cytocidal contra lymphocytos sanguine ab 11 patientes con chronic leucemia lymphocytic. Le titros del seros immun variava inter 1:42 e 1:530, con un titro medie de 1:100. Le seros manifestava toxicitate minime o nulle toxicitate contra lymphocytos ab subjectos normal, con un titro de >1:10 in 13 de 18 casos. Un simile toxicitate differential contra normal e leucemic lymphocytos esseva observate con sero de ratto anti lymphocytos canin. Tamen, sero o globulina de porco de India e de cavallo anti lymphocytos canin esseva igualmente toxic contra normal e leucemic lymphocytos human. Lymphocytos leucemic incubate con sero de conilio anti lymphocytos canin insimul con complemento de conilio mostrava precoce alterationes nucleo-degeneratori e vacuolos paranucleari. Le cytologic alterationes degeneratori suggestionava que le nucleo o le membrana nucleari es possibilemente le prime sito de action del sero immun.

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