CONCISE REPORT

Lymphocyte-Mediated Lysis of Antibody Coated Human Red Cells in the Presence of Human Serum

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When peripheral blood lymphocytes and human red cells coated with IgG were incubated in vitro in culture medium, antibody-dependent lymphocyte-mediated lysis was observed. This lysis was markedly inhibited by the addition of purified monoclonal IgG1 (1000 µg/ml) to the culture medium. In contrast, lysis by lymphocytes of sensitized red cells in the presence of undiluted human serum was equal to or greater than lysis in medium alone, even in the presence of IgG1 at 1000 µg/ml, despite the high concentration of IgG in human serum (6000–19,000 µg/ml). Serum heated to 56°C for 30 min also restored lysis in the presence of IgG1. When serum was separated into three fractions by passage through a Sephadex G-200 column, the third fraction, which contained proteins with a molecular weight of less than 100,000 d (but neither of the other two fractions nor purified human albumin), restored lymphocyte-mediated lysis in the presence of IgG1.

The destruction in vivo of red cells coated with IgG antibodies appears to be mediated in part by effector cells in the immune system capable of partial1,2 or total3 phagocytosis or direct cytolysis4 of sensitized red cells. Monocytes and macrophages have been considered effectors of red cell destruction for many years.1 More recently it has been established that lymphocytes also may mediate destruction of human red cells coated with antibody.4-7 However, destruction by all these effectors is markedly inhibited by unbound fluid-phase IgG,1,7-9 and this inhibition has engendered doubt about the role of these effectors in vivo, since the amount of IgG that effectively inhibits target cell destruction in vitro is far less than is present in serum.7-9 In these experiments we demonstrated that in contrast to the destruction mediated by monocytes, the destruction mediated by lymphocytes is inhibited by fluid-phase IgG but not by serum. A protein component is present in serum that augments lysis of sensitized red cells by lymphocytes despite high concentrations of unbound IgG.

MATERIALS AND METHODS

Buffers and Reagents

Veronal buffered saline (VBS), Alsever’s solution, 0.015-M EDTA in distilled water (0.015-M EDTA), and 0.015-M EDTA in VBS (0.015-M EDTA + VBS) were prepared as previously.

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described. RPMI-1640 and fetal calf serum were obtained from Grand Island Biological Co., Grand Island, N.Y. RPMI-1640 with 5% fetal calf serum and 25-mM HEPES (Sigma Chemical, St. Louis, Mo.) adjusted to pH 7.2 was used as medium.

Chromium Labeling of Human Red Cells

Red cells obtained after informed consent from a single normal volunteer (O Rh-positive) were used in these experiments. Blood collected under sterile conditions and stored in Alsever’s solution for up to 4 wk prior to use labeled satisfactorily with 51Cr with less than 3% spontaneous Cr release during the 2-hr incubation. The procedure for chromium labeling and determination of red cell radioactivity per 10^7 cells has been described in detail previously. Chromium-labeled or unlabeled red cells at a concentration of 2-4 x 10^7 cells/cc in VBS were incubated with an equal volume of purified IgG fraction with a high titer of anti-D (RhD) activity obtained from hyperimmune human volunteers (Rhogam, Ortho Pharmaceuticals, Raritan, N.J) at a dilution of 1:50 for 1 hr at 37°C. Unbound antiserum was then removed by washing the cells with VBS four times, and the cells were resuspended at 5 x 10^7 cells/cc. Cells coated in this manner have been shown previously to be coated with 5000-10,000 molecules of IgG antibody per red cell.

Preparation of Fresh Human Serum

From 30 to 50 cc of venous blood were collected from normal donors after obtaining informed consent. Red cells and leukocytes were separated by centrifugation (vide infra) at 12,000 g at 4°C for 2 min, and serum was collected after incubation at 37°C for 30 min to allow coagulation to reach completion. The serum was either used immediately or stored at −70°C for up to 2 wk prior to use. Before use, HEPES (25 mmoles/liter) was added, and the serum was adjusted to pH 7.2.

Preparation of Purified Lymphocyte Suspension

The red cell/leukocyte mixture obtained after removal of plasma as described previously was diluted with 0.015-M EDTA + VBS and purified lymphocyte preparation prepared using Ficoll-Hypaque density centrifugation and carbonyl iron treatment as described previously. Purified lymphocyte preparations consistently contained less than 1% monocytes, as detected by morphologic criteria or by esterase staining.

Antibody-Dependent Lymphocyte-Mediated Red Cell Lysis

51Cr release was measured after incubation of labeled red cells with or without antibody coating with lymphocytes. Fifty microliters of a suspension of antibody-sensitized washed red cells or unsensitized red cells suspended in medium or in serum at a concentration of 5 x 10^7 cells/ml and 100 µl of suspension of lymphocytes at a concentration of 1 x 10^7 cells/ml were mixed in 12- x 75-mm plastic tubes in triplicate (Fisher Scientific, Pittsburgh, Pa.) and were incubated in a moisture incubator at 37°C for 2 hr. In some experiments the cell suspension was pelleted by centrifugation for 30 sec at 1000 g prior to incubation. Following incubation, 0.85 ml of EDTA + VBS was added, and each tube was centrifuged at 1000 g for 5 min to sediment unlysed cells. Five hundred microliters of supernatant fluid were then carefully removed from each tube, and the radioactivity was determined using a gamma emission spectrometer. Chromium release from antibody-coated red cells incubated without leukocytes and chromium release from uncoated red cells incubated with leukocytes were measured in each experiment. Since leukocyte-mediated chromium release from uncoated red cells was consistently absent, antibody-dependent leukocyte-mediated red cell lysis was calculated using the formula:

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\text{Antibody-dependent lymphocyte-mediated red cell lysis (cells lysed} \times 10^{-7}) = \frac{51\text{Cr release from antibody-coated red cells plus lymphocytes} - 51\text{Cr release from antibody-coated red cells without lymphocytes}}{\text{radioactivity/10^7 red cells}} \times 2
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The standard error in red cells lysed × 10^-7/tube was calculated by dividing the standard error of the mean of the numerator of this equation by the denominator.
Preparation of IgG and Determination of Inhibition of Lysis or Phagocytosis by IgG1

Purified IgG1 was prepared by DEAE cellulose purification in 0.005-M phosphate buffer (pH 8.0) of serum obtained from a patient with multiple myeloma that contained monoclonal IgG1 at a concentration greater than 12 g/100 ml. The IgG solution was concentrated and stored in small portions for future use. In experiments measuring inhibition of lysis, red cell and leukocyte suspensions were prepared in medium with and without IgG1 (1000 μg/ml) and were then assayed for lymphocyte-mediated red cell destruction using the methods described previously.

RESULTS

Antibody-Dependent Lymphocyte-Mediated Lysis in Medium and in Serum

Antibody-dependent lymphocyte-mediated lysis detected by ⁵¹Cr release of red cells coated with anti-D was compared in the presence of medium alone, medium with added purified IgG1 (1000 μg/ml), and fresh autologous serum. Purified lymphocytes from 9 normal donors were used in 12 experiments (Fig. 1). The geometric mean lysis in medium was 2.9 × 10⁴ red cells per tube, and when incubation was performed under identical conditions in fresh serum. In 9 of 12 experiments, lysis was greater in serum than in medium alone.

In contrast, when IgG1 at 1000 μg/ml was added to medium, the geometric mean lysis was 0.06 × 10⁴ cells lysed per tube in 7 experiments (Fig. 1). Centrifugation markedly increased lysis in the presence of IgG1 in all 3 experiments to an average lysis of 4.0 × 10⁴ red cells lysed per tube. In contrast, when red cell/lymphocyte suspensions in medium without IgG or in serum were subjected to centrifugation prior to incubation, lysis was minimally increased.

Fig. 1. Comparison of lymphocyte-mediated lysis of human red cells coated with anti-D in the presence of medium alone, autologous serum, and medium with IgG1 (1000 μg/ml). Although purified IgG markedly inhibited lysis, lysis in serum was greater than that in medium without IgG. Lysis in medium with IgG1 could be restored to levels observed in the absence of IgG1 by centrifugation of the red cell/lymphocyte suspension prior to incubation.
Effect of Serum on Inhibition of Lymphocyte-Mediated Lysis by Purified Monoclonal IgG1

Since lysis in the presence of serum exceeded lysis in medium containing IgG1 at 1000 μg/ml, the effect of serum on inhibition by IgG1 was assessed by adding graded dilutions of serum to lymphocyte/red cell mixtures at a constant concentration of purified IgG1 (1000 μg/ml). Lysis in undiluted serum with added IgG1 was identical to that observed in serum without added IgG1. The augmentation of lysis by serum was observed at dilutions of 1:2 and 1:4 but not 1:8 (Fig. 2, open circles).

Serum heated for 30 min at 56°C also augmented lysis by lymphocytes. Normal serum was passed through a Sephadex G-200 column; then each of the three major protein peaks was concentrated by vacuum dialysis to the original volume of the serum, was dialyzed against saline, and was assessed for its capacity to augment lymphocyte-mediated lysis in the presence of unbound IgG1 (Fig. 3). Only the third peak significantly increased lysis above that observed in the presence of IgG1 alone. Purified human albumin did not augment lysis markedly in the presence of added IgG1.

DISCUSSION

The inhibition of interaction between lymphocyte effectors and antibody-coated targets (red cells) by fluid-phase IgG is thought to be the result of competition between membrane-bound antibody and fluid-phase IgG for binding to a limited number of receptors for the Fc portion of IgG on the leukocyte membrane. The complex between the membrane-bound IgG and the Fc receptor is the basis for immune adherence and is probably a prerequisite for destruction of the target red cells by the lymphocytes. Hence, competitive displacement of membrane-bound by
fluid-phase IgG results in inhibition of both adherence and destruction.\textsuperscript{13} The inhibition of monocyte- and macrophage-mediated destruction of antibody-coated targets\textsuperscript{18,19} by IgG1 or serum is presumably on this basis.

In the presence of serum, monocyte-mediated lysis is virtually but not completely inhibited.\textsuperscript{14} In marked contrast, lysis by lymphocytes of antibody-coated red cells is often augmented by serum. This augmentation appears to be caused by a protein of low molecular weight that is different from albumin. Augmentation of lysis occurs with serum heated to 56°C for 30 min, and these conditions are known to destroy factor B, C2, and perhaps other components of the complement system. The precise nature of this component that augments lymphocyte-mediated lysis is currently under investigation.

The finding that monocyte-mediated hemolysis is inhibited by serum, whereas lymphocyte-mediated hemolysis is augmented, suggests that lymphocytes may play a more important role in the destruction in vivo of red cells of patients with immune hemolysis than has previously been appreciated.

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