51Cr Release Microassay for Measuring Cell-Mediated Immunity in Chickens

A. I. RADWAN,† G. M. BUENING,* AND R. W. LOAN

Department of Veterinary Microbiology, College of Veterinary Medicine, University of Missouri, Columbia, Missouri 65201

Received for publication 11 September 1974

A microcytotoxic assay, based on the release of 51Cr from chicken erythrocytes by sensitized chicken lymphoid cells in the presence of the specific antigen, purified protein derivative (PPD), is described. The percentage of antigen-specific lysis was dependent on the lymphoid cell concentration, antigen concentration, time of incubation, and incubation temperature. Maximum antigen-specific lysis of nonspecific target cells was induced with a spleen-to-target cell ratio of 100:1 and with a peripheral blood lymphoid-to-target cell ratio of 1,000:1 in the presence of 100 μg of PPD per ml over a 20-h incubation period. As the concentration of PPD was increased from 25 to 200 μg/ml the specific lysis increased. Incubation for 40 h resulted in higher antigen-specific lysis than incubation for 20 h. Incubation at 41 C produced significantly higher (P < 0.05) antigen-specific cytotoxicity than incubation at 37 C.

Lymphoid cells are known to have effector functions in certain tissue-damaging reactions such as delayed hypersensitivity, autoimmunity, allograft rejection, and some forms of tumor rejection. Several methods for measuring cell-mediated cytotoxicity have been developed based on observable or measurable reactions such as destruction of cell monolayers (1), reduction in the number of attached cells (2), inhibition of colony formation (3), and isotope release from labeled target cells (4).

Chromium-51 is a useful label for the quantitative determination of cell-mediated lysis. The 51Cr label, which is reduced during binding to protein and other cell constituents, is not reutilized by the cells (5). Perlmann et al. (6) and Granlund et al. (7), using macrocytotoxic assay systems, reported that the release of 51Cr from chicken erythrocytes (ChRBC) could be used to measure phytohemagglutinin-induced lymphoid cell cytotoxicity. This procedure was subsequently adapted by Granlund and Loan (8) to measure antigen-induced lymphoid cell cytotoxicity.

The present investigation was undertaken to develop a more economical and convenient in vitro microcytotoxic assay for the demonstration and quantitation of cell-mediated immunity in chickens.

MATERIALS AND METHODS

Sensitization of chickens. White leghorn chickens, 5 to 6 months of age, were sensitized to tuberculin by intraperitoneal injection of 1.0 ml Freund complete adjuvant with 5 x concentration (5 mg/ml) of Mycobacterium tuberculosis H37Ra (Difco, Detroit, Mich.) emulsified with an equal volume of Puck saline G. Control birds were injected with 1.0 ml of Freund incomplete adjuvant emulsified with an equal volume of Puck saline G. Two weeks after injection, the chickens were tested for delayed reactivity by intradermal injection in the wattle of 10 μg (0.1 ml) of tuberculin purified protein derivative (PPD) (Park, Davis and Co., Detroit, Mich.). The reactions were measured as the percentage of increase in wattle thickness by using calipers. A 100% or more increase in wattle thickness after 24 h was considered a strong positive reaction (10). Lymphoid cells from these chickens were collected and used for cytotoxic tests 5 weeks after a strong positive wattle test was elicited.

Media. Medium RPMI-1640 with N-2-hydroxyethyl-piperazine-N'2-ethane-sulfonic acid buffer was supplemented with 10% fetal bovine serum (inactivated at 56 C for 60 min), 50 U of penicillin per ml, 50 μg of streptomycin per ml, and 10 U of mycostatin per ml. Puck saline G was used without supplementation or with 10% fetal bovine serum, 50 U of penicillin per ml, 50 μg of streptomycin per ml, and 10 U of mycostatin per ml (supplemented Puck saline G).

Preparation of spleen cells. Chickens were exsanguinated by cardiac puncture. The spleens were removed aseptically and placed in cold Puck saline G in an ice bath. Spleen cell suspensions were prepared by mincing the splenic tissue (after removal of the capsule) with scalpels on a 40-mesh, stainless-steel wire screen. The lymphoid cells were washed from the spleen fragments with supplemented Puck saline G. The resulting suspension was poured through an 80-mesh, stainless-steel wire screen and then through eight layers of sterile gauze. The cell suspension was centrifuged for 5 min at 55 x g to pellet the erythro-
cytes. The supernatant fluid was then centrifuged at 225 \times g for 10 min. The lymphoid cell pellet was washed four times with supplemented Puck saline G and adjusted to 2 \times 10^5 (unless otherwise indicated) trypan blue, excluding small lymphoid cells, per ml in RPMI-1640 culture medium.

**Preparation of lymphoid cells from peripheral blood.** The blood obtained by cardiac puncture was drawn into syringes containing 5.0 ml of Puck saline G with sufficient preservative-free heparin to give a final concentration of 5 U of whole blood per ml. The blood was immediately mixed with an equal volume of 6% dextran in normal saline and centrifuged at 55 \times g for 5 min. The supernatant fluid was removed and centrifuged at 225 \times g for 10 min. The lymphoid cells were washed four times with saline G and then adjusted to 2 \times 10^7 cells/ml (unless otherwise indicated) in RPMI-1640 medium.

**Preparation of target cells.** Chicken blood (0.2 ml) was obtained from normal birds (3 to 4 months old) by venipuncture diluted 1:20 in Puck saline G (with serum and antibiotics), and a 0.1-ml sample was mixed with an equal volume of sodium chromate (Na\textsubscript{4}CrO\textsubscript{4}) from Amersham Searle Co., Arlington Heights, Ill. The specific activity of the \textsuperscript{67}Cr was 1 mCi/ml (4.4 to 7.6 \muCi/ml). The amount of sodium chromate added was adjusted to compensate for radioactive decay. The mixture was incubated for 20 h at 37 C on a tissue culture rocker. Prior to use in the assay the labeled ChRBC were washed three times with supplemented Puck saline G and adjusted to 2 \times 10^5 cells/ml in RPMI-1640 medium.

**Cytotoxic microassays.** The procedure of Granlund et al. (4) was modified for use with flat-bottomed, plastic tissue culture microtiter plates (Cooke Laboratory Products, Alexandria, Va.). The plates were placed in a water bath (Bello Equipment, Vine-land, N.J.), set at two reciprocations per minute, and incubated at 37 C (unless otherwise indicated in a humidified CO\textsubscript{2} incubator. Micropipettes with sterile disposable tips (Centaur Chemical Co., Danbury, Conn.) were used to deliver target cells, lymphoid cells, antigen, and medium to the wells of the microtiter plates.

In test wells, 0.1 ml of lymphoid cells (4 \times 10^9 cells/ml unless otherwise indicated) and 0.1 ml of labeled target cells (2 \times 10^5 cells/ml) were mixed, and 0.05 ml of RPMI-1640 medium with or without 100 \muCi of PPD per ml (unless otherwise indicated) was added. A constant volume of 0.25 ml/well was maintained for all tests. Nonspecific cytolysis by PPD was ruled out by testing target cells with sensitized and nonsensitized lymphoid cells in the presence and absence of PPD. To determine the total releasable label (100%), 0.1 ml of target cells was incubated with 0.15 ml of 3.0% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.). Triplicate samples of all treatments and controls were tested.

After 20 or 40 h of incubation, 0.1 ml of the supernatant fluid in each well was carefully removed (without disturbing the cells), and the radioactivity was measured in an automated sodium iodide crystal gamma counter (Nuclear Chicago, Des Plaines, Ill.). Average counts per minute for target cells usually exceeded 2,500. Stulting and Berke (9) indicated that a simple formula for expressing cytotoxicity is preferred. The percentage of antigen-specific lysis was calculated as equal to: (average counts per minute of \textsuperscript{67}Cr released in the presence of sensitized lymphoid cells and PPD) – (average counts per minute of \textsuperscript{67}Cr released in the presence of sensitized lymphoid cells without PPD)/total counts per minute of \textsuperscript{67}Cr released from target cells \times 100.

**Evaluation of reagents for microcytotoxic assay.** Preliminary experiments were conducted to standardize the reagents for the microcytotoxic assay. Fetal calf serum lots were pretested in vitro on chicken lymphoid cells stimulated with phytohemagglutinin-protein as described by Granlund et al. (7). The procedures of Granlund et al. (4) were modified in the following manner. Medium RPMI-1640 was substituted for Trowell T8 medium. The ChRBC were incubated with \textsuperscript{67}Cr for 24 h instead of 3. h. The increase in incubation time resulted in a threefold increase in the counts per minute of isotope uptake by the target cells. The spontaneous release of \textsuperscript{67}Cr from labeled ChRBC in the presence of lymphoid cells without antigen varied both with time and temperature of incubation. For example, after 2 h of incubation at 37 C, approximately 6% of the label is released. After 40 h of incubation at 41 C, approximately 50% of the label is released. However, by using the above formula to calculate the percentage of antigen-specific lysis, spontaneous release is compensated for and all values can be compared. This formula does not inflate calculated values as spontaneous release increases (1).

**RESULTS**

**Lymphoid-to-target cell ratio.** Different ratios of lymphoid cells to target cells were tested to determine the maximum cytotoxic response. With sensitized spleen cells, the percentage of antigen-specific lysis increased gradually when the ratio was decreased from 1,000:1 to 62.5:1. However, at the 62.5:1 ratio, the standard deviation increased. The cytotoxicity of sensitized peripheral blood lymphoid cells was higher at a lymphoid cell-target cell ratio of 1,000:1 than at lower ratios (Fig. 1). For practical reasons, this was the highest ratio tested. For all subsequent experiments, a spleen-to-target cell ratio of 100:1 was used. For peripheral blood, a lymphoid-to-target cell ratio of 1,000:1 was employed.

**Effect of different concentrations of antigen.** The reactivity of sensitized lymphoid cells was tested in the presence of various dilutions of PPD. The percentage of antigen-specific lysis gradually increased with increasing concentrations of PPD (Fig. 2), indicating a dose-response relationship.

**Effect of temperature on cytotoxicity.** The effect of two different temperatures on cytotoxicity was investigated. Parallel tests were incubated at 37 and 41 C for 20 h in the presence of.
100 \mu g of PPD per ml (Table 1). With sensitized spleen cells, target cell lysis was increased approximately 100\% by incubation at 41 C as compared to 37 C. With sensitized peripheral blood lymphoid cells, target cell lysis after incubation at 41 C was over 300\% higher than at 37 C. Both of these differences were statistically significant at the 5\% level using the Student’s t test of significance.

**Effect of incubation period on cytotoxicity.**

The effect of prolonged incubation at 37 C was investigated (Table 2). The percentage of antigen-specific target cell lysis induced by sensitized lymphoid cells was found to be increased by prolonged incubation at 37 C in the presence of 200 \mu g of PPD per ml. Target cell lysis induced by spleen cells was more extensive during the first 20 h of incubation than that induced by peripheral blood lymphoid cells during the same period. Sensitized lymphoid cells from expresser lymphoid cells, O; sensitized peripheral blood lymphoid cells, x.

![Graph showing reactivity of sensitized chicken lymphoid cells](image)

**Table 1. Effect of incubation temperature on percentage of antigen-specific lysis by M. tuberculosis-sensitized chicken spleen and peripheral blood lymphoid cells in the presence of 100 \mu g of PPD per ml**

<table>
<thead>
<tr>
<th>Source of lymphoid cells</th>
<th>% Specific lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen of normal chicken (1)</td>
<td>1.57 \pm 0.72%</td>
</tr>
<tr>
<td>Spleen of sensitized chicken (4)</td>
<td>26.66 \pm 1.62%</td>
</tr>
<tr>
<td>Peripheral blood of normal chicken (1)</td>
<td>2.30 \pm 0.66</td>
</tr>
<tr>
<td>Peripheral blood of sensitized chicken (4)</td>
<td>16.15 \pm 0.96%</td>
</tr>
</tbody>
</table>

* Number in parentheses indicates number of chickens.
* Expressed as mean of three determinations for each subject \( \pm \) standard deviation.
* Values are significantly different from normal \( < 0.0001 \).

**Table 2. Effect of incubation period at 37 C on percentage of antigen-specific lysis by M. tuberculosis-sensitized chicken spleen and peripheral blood lymphoid cells in the presence of 200 \mu g of PPD per ml**

<table>
<thead>
<tr>
<th>Source of lymphoid cells</th>
<th>% Antigen-specific lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen from sensitized chicken (5)</td>
<td>51.60 \pm 6.23%</td>
</tr>
<tr>
<td>Peripheral blood from sensitized chicken (5)</td>
<td>20.06 \pm 2.49</td>
</tr>
</tbody>
</table>

* Number in parentheses indicates number of chickens.
* Values are expressed as grand mean \( \pm \) standard deviation.
cells from peripheral blood lysed target cells to a greater extent during the period between the 20th and 40th hour of incubation.

**DISCUSSION**

Granlund et al. (4) reported that cell-mediated cytotoxicity in the chicken was independent of bursal lymphocytes and dependent upon the presence of thymic lymphocytes. Synthesis of deoxyribonucleic acid was not necessary for cytotoxicity, but protein synthesis was required. This micro-modification of the procedure of Granlund et al. (4) has a number of advantages over the macro-procedure. The total number of lymphoid cells required for an assay was greatly reduced. This allowed investigations to be conducted using the peripheral blood as the source of lymphoid cells; thus, repeated sampling could be done on the same birds. The ¹¹¹Cr microassay was quantitative and objective.

The basis for the cytotoxic effect on nonspecific labeled targets could be a “bystander effect” due to a lymphokine or a direct effect. Previous work by Granlund and Loan (5) with the macrocytotoxicity assay system would suggest a bystander effect. They (5) found that when ChRBC were incubated with PPD and washed prior to use in the assay, no significant antigen-specific lysis was detected. However, when the same sensitized lymphoid cells were incubated with ChRBC in the continuous presence of PPD, antigen-specific cytotoxicity was observed.

In conducting cytotoxic assays, it is important to label the ChRBC overnight with ¹¹¹Cr to obtain satisfactory levels of isotope incorporation. The use of nonspecific fowl erythrocyte target cells has several advantages. The ChRBC’s are easily obtained and are very susceptible to cell-mediated lysis. The system can be readily adapted to measure cell-mediated immunity in different species and to a number of different antigens.

Results of this study indicate that it may be necessary to modify the procedure according to the source of lymphoid cells. For example, the best ratio of sensitized spleen-to-target cells appeared to be approximately 100:1, whereas with peripheral blood lymphoid cells the best ratio may be as high as 1,000:1 (Fig. 1). This observation indicated that there were a smaller number of sensitized cells in the peripheral circulation. A satisfactory concentration of PPD was 100 μg/ml with both the spleen and peripheral blood assays. Although slightly higher antigen-specific lysis occurred with PPD at 200 μg/ml, this was not statistically significant (Fig. 2).

The avian species has a normal body temperature of 41 C. For this reason the effect of various temperatures on antigen-specific cell-mediated lysis was investigated. Increasing the temperature of incubation from 37 to 41 C significantly increased the antigen-specific lysis in both the sensitized spleen and peripheral blood assays (P < 0.05). It is reasonable that avian lymphocytes may have optimal metabolic activity at this normal avian body temperature.

The length of the incubation period also influenced the level of antigen-specific lysis. With sensitized spleen cells increasing the incubation period from 20 to 40 h did not significantly increase the level of lysis (P < .05). With peripheral blood as the source of sensitized cells, increasing the incubation period doubled the antigen-specific lysis (Table 2).

The percentage of PPD antigen-specific lysis reported in this study with the ¹¹¹Cr microcytotoxic assay is comparable and in most cases higher than that reported for the macrocytotoxic procedure of Granlund and Loan (5). A suggested protocol for the ¹¹¹Cr microcytotoxic assay using avian lymphocytes sensitized to Mycobacterium tuberculosis would be as follows. With spleen lymphoid cells use a ratio of 100 lymphoid cells to one target cell. 100 μg of PPD per ml, and incubate at 41 C for 20 h. With peripheral blood as the source of sensitized lymphoid cells, a lymphoid-to-target cell ratio of 1,000:1 should be used with 100 μg of PPD per ml. The data suggest that tests should be incubated at 41 C for 40 h.

**ACKNOWLEDGMENTS**

This investigation was supported by Public Health Service grant no. 5 RO1 CA 13134 from the National Cancer Institute. We wish to express our appreciation to Patsy Kaiser for her excellent technical assistance and to D. F. Hutcheson and L. A. Selby for assistance in statistical analysis of the data.

**LITERATURE CITED**

6. Hellstrom, I. 1967. A colony inhibition (CI) technique for...