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Direct cytotoxicity against chicken erythrocytes in mice

I. FUNDAMENTAL NATURE OF T CELL-MEDIATED CYTOTOXICITY

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Summary. Immune responses were examined after immunization with chicken erythrocytes (CRBC) in mice. Cytotoxicity of spleen cells was assessed by the release of ^{51}Cr from labelled target cells. (1) At early stages (day 4–7) after primary intraperitoneal immunization, direct cytotoxicity of spleen cells was raised efficiently in C57BL/6 and AKR mice, but not in C3H/He, SL and DDD mice. Delayed hypersensitivity and antibody production were raised to almost the same extent in all the strains at such periods. (2) Effector cells in direct cytotoxicity were θ-positive and IgG-positive, and glass-nonadherent and Nylon wool column-adherent. Effector cells in antibody-dependent cell-mediated cytotoxicity in the presence of antibody to CRBC were eliminated by treatment with anti-IgG serum, but not by treatment with anti-θ serum. (3) Cytotoxicity and antibody production were raised efficiently after intraperitoneal or intravenous immunization, but not after footpad immunization. On the other hand, delayed hypersensitivity developed most efficiently after footpad immunization.

INTRODUCTION

Various immunological phenomena attributable to humoral or cellular immunity have been shown to be raised concurrently in the same individuals after antigenic stimulation. Identification of each phenomenon as an independent entity and analysis of their mutual relationships have been needed for further advances in immunological study. A study in our laboratory using sheep erythrocytes (SRBC) has shown that effector cells of tuberculin-type of hypersensitivity, those of Jones-Mote type hypersensitivity and helper cells in antibody production belong to distinct subpopulation of differentiated thymus-derived (T) lymphocytes and each of them can be generated independently under some conditions (Ohmichi, Nomoto, Yamada & Takeya, 1976). Cytotoxic lymphocytes are generated usually by immunization with allogenic skin grafts or tumour grafts. In such systems, cytotoxicity can be induced and assessed readily, but antibody production and delayed hypersensitivity are rather difficult to raise efficiently and assess exactly. Thus, relationships of cytotoxic lymphocytes to other types of differentiated T cells have not yet been understood. A new experimental system, in which cytotoxic activity, delayed hypersensitivity and antibody production can be raised and assessed readily, has been needed for studies on such a problem.

In order to analyse the mutual relationships in the developmental stage of various immunological phenomena, experiments have to be conducted on the known basis of genetic control. Genetic control of immune response has been studied mainly on protein antigens, heterologous erythrocytes and synthetic

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polypeptides as described in several reviews (McDevitt & Benacerraf, 1959; Benacerraf & McDevitt, 1972; McDevitt & Landy, 1972; Shreffler & David, 1975). Many of such controls have geen ascribed to the genes which regulate the antigen-specific functions of T cells and are inherited as dominant traits. Such antigens can readily induce antibody production or delayed hypersensitivity, but not cytotoxicity. Consequently, cytotoxicity has not yet been understood satisfactorily from the viewpoint of genetic control.

We have found that cytotoxicity against chicken erythrocytes (CRBS) can be raised in mice and the cytotoxic activity depends upon T cells as presented below. This system appears to be valuable for the analytical studies on mutual relationships of various immunological phenomena and a genetic control of cytotoxicity, since not only cytotoxicity but also antibody production and delayed hypersensitivity can be assessed very easily against such an antigen. Furthermore, immunization can be carried out as quantitatively as in the systems with protein antigens or synthetic polypeptides.

MATERIALS AND METHODS

Animals

Female and male mice of inbred C57BL/6, C3H/He, AKR, SL and DDD strains were obtained from the Breeding Unit of Kyushu University School of Medicine. Ten-week-old male mice were used as the standard hosts for experiments and the same protocols were repeated two or three times. In most experiments, except for those conducted to study cytotoxicity, each group consisted of eight animals. Averages of repeated experiments were given in most of experimental protocols.

Antigens

Fresh CRBC were obtained from an adult female by a venipuncture and used as an immunogen and a target. Kidney cells of new-born chicken and EL-4 leukaemia cells of C57BL/6 origin were used as immunogens and targets in some experiments.

Immunization

Immunization with 1×10^8 CRBC in saline via the intraperitoneal route was chosen as a standard protocol. The spleen served as the standard source of effector cells. Cytotoxicity of lymphoid cells, antibody titres and delayed footpad reaction were assessed at various times after immunization. Varying numbers of CRBC injected via the intraperitoneal or intravenous routes were examined for their efficiency in inducing immune responses. In some experiments, mixtures of CRBC suspension and an oil phase containing 8·5 parts Drakeol Six and 1·5 parts Arlacel A with mycobacteria (Freund's complete adjuvant, FCA) or without mycobacteria (Freund's incomplete adjuvant, FIA) were used for immunization. Each mouse received 1×10^8 CRBC in 0·2 ml of the mixtures. In some experiments, 1×10^7 chicken kidney cells and 1×10^6 EL-4 cells were injected intraperitoneally and subcutaneously, respectively.

Effector cells

Mice were bled by cutting the femoral arteries and the spleens and lymph nodes were removed. The lymphoid tissues were squeezed with two glass slide in Hanks' balanced salt solution (HBS). The cell suspensions were passed through a layer of gauze to remove residual large fragments. Peritoneal cells were obtained without the use of any irritant. The cell suspensions were adjusted to the desired concentration after counting viability by the trypan blue exclusion method.

Target cells

Labelling of CRBC, kidney cells or EL-4 cells with radioactive chromium was carried out according to Perlman & Perlman (1970). 100 μCi of Na_2CrO_4 (^{51}Cr) was added to 1×10^8 cells in 3 ml of Tris-buffered Hanks' balanced salt solution (THBS) containing 10% foetal calf serum (FCS). After incubation at 37° for 1 h, the cells were washed three times with 10 ml of THBS containing 10% FCS. After the last washing, the labelled cells were resuspended in TC199 containing 10% FCS.

Assay of direct cytotoxicity

2×10^7 effector cells were mixed with 10^6 labelled CRBC, kidney cells or EL-4 cells in 2 ml of TC199 containing 10% FCS in roller tubes. The tubes were incubated at 37° for 6 h in an atmosphere containing 5% CO_2 and 95% air, unless otherwise noted. After centrifugation at 1000 rev/min for 10 min, the radioactivity in the supernatant fluid was assessed by a well type gamma counter. When labelled CRBC were used as target, spontaneous release of ^{51}Cr in the absence of lymphoid cells was negligible.

Cytotoxicity was expressed as the per cent release of radioactivity calculated by the following formula:
Percent cytotoxicity =

$$\frac{\begin{pmatrix}\text{c.p.m. in super-}\\ \text{natant in the}\\ \text{presence of effector}\\ \text{cells}\end{pmatrix} - \begin{pmatrix}\text{c.p.m. in super-}\\ \text{natant in the}\\ \text{absence of effector}\\ \text{cells}\end{pmatrix}}{\begin{pmatrix}\text{total release after}\\ \text{treatment with}\\ \text{distilled water}\end{pmatrix} - \begin{pmatrix}\text{c.p.m. in super-}\\ \text{natant in the}\\ \text{absence of effector}\\ \text{cells}\end{pmatrix}} \times 100$$

Effector cells were pooled from three or four animals of each group and the same protocols were repeated two to four times. Averages of the repeated experiments were presented in most of the experiments.

Assay of antibody dependent cell-mediated cytotoxicity
The ability of sera to induce antibody dependent cell-mediated cytotoxicity (ADCC) was assessed according to Perlman & Perlman (1970). Normal spleen cells of C57BL/6 mice and labelled CRBC were used as effector cells and target cells, respectively. Sera obtained from immunized C57BL/6 mice were diluted 4-fold and 0·2 ml of the diluted sera was added to 2 ml of culture medium. After incubation at 37° for 6 h, radioactivity released in the culture supernatant was assessed as described above.

Detection of plaque-forming cells
Antibody plaque-forming cells (PFC) were counted according to Jerne & Nordin (1963) after minor modifications as described in detail elsewhere (Nomoto, Mashiba & Takeya, 1972). 2×10^6 spleen cells were plated on a plastic dish (60 × 10 mm) after mixing with 20% CRBC in 0·8% soft agar. In order to remove an anti-complement effect of agar, DEAE dextran was added to soft agar at a final concentration of 0·13 mg/ml.

Titration of haemagglutinins (HA)
One-tenth ml of blood was obtained by a retro-orbital venipuncture at various times after immunization. The blood was added to 0·15 ml of saline to give an approximate dilution of 1/4. The diluted sera were used as the starting specimens for titration after heat-inactivation at 56° for 30 min. In order to determine the titres of 2-mercaptoethanol (2-ME) resistant antibodies, sera were diluted with saline containing 2-ME to obtain a final concentration of 0·1 M and incubated at 37° for 30 min before the addition of a test antigen. Titration was carried out with the micro-method and titres were expressed as $\log_2$.

Footpad reaction
50 μl of saline containing 1×10^8 CRBC were injected into the subcutaneous tissue on the plantar surface of the right hind footpad. 50 μl of saline without CRBC were injected into the left hind footpad as control. Swelling of the footpad was measured 24 and 48 h later. Differences in the thickness between the right and left hind footpads were presented as the degrees of reaction. Swelling of 0·1 mm was expressed as one unit.

Treatment with anti-θ serum and anti-IgG serum
Anti-θ (Thy 1.2) and anti-θ (Thy 1.1) sera were prepared, respectively, in AKR and C3H/He mice according to Reif & Allen (1964). Rabbit anti-mouse IgG serum was purchased commercially (Institute for Medical Biology, Nagoya, Japan). Elimination of T cells was carried out by the treatment of spleen cells with 1/4-dilution of anti-θ serum and 1/2-dilution of rabbit complement (C) at 37° for 30 min. Elimination of B cells was carried out by the treatment of spleen cells with 1/10-dilution of anti-IgG serum and 1/2-dilution of rabbit complement at 37° for 30 min. Spleen cells treated with normal serum and rabbit complement were used as controls. Viable cells were counted before the treatment and adjusted to the desired concentration. These cells were used as effector cells after washing without re-adjustment. Mitogenic responses to Con A and LPS were diminished, respectively, by the treatment with anti-θ serum and anti-IgG serum as reported by Niederhuber, Frelinger, Dine, Shoffner, Dugan & Shreffler (1976) and Kumar, Caruso & Bennett (1976).

Fractionation of lymphocytes adherent to glass surface or Nylon wool column
In order to eliminate cells adherent to the surface of tissue culture dishes (glass-adherent cells), spleen or peritoneal cells were incubated on each of four surfaces of a culture bottle for 30 min and free cells (glass-nonadherent cells) were harvested. The glass-nonadherent cells did not include detectable macrophages. Elimination of Nylon wool column-adherent

cells was carried out according to Julius, Simpson & Herzenberg (1973). 2×10^8 spleen cells in 2 ml were loaded onto a previously rinsed column and allowed to incubate at 37° for 30 min. Nonadherent cells were then gently washed off with 20 ml of cold medium. The Nylon wool was removed from the column, placed into a beaker and stirred gently with forceps to elute adherent cells. These populations were used, respectively, as Nylon wool column-nonadherent and adherent cells. After fractionation, each cell population was counted by trypan blue exclusion method and adjusted to the desired concentration. The cell suspensions were subjected to treatments with anti-θ serum or anti-IgG serum and complement after adjustment.

Detection of θ-antigen and IgG antigen on the surface of lymphocytes

θ-antigen and IgG-antigen on the surface of lymphocytes were observed by the fluorescent antibody technique according to Möller (1961). B cells were counted by a direct method with fluorescein-conjugated rabbit anti-mouse IgG serum (Behringwerke, West Germany). The numbers of T cells were calculated by subtracting the numbers of B cells from the numbers of cells positively stained by an indirect method with anti-θ serum and fluorescein-conjugated rabbit anti-mouse IgG serum.

RESULTS

Time course of ^{51}Cr-release from labelled target cells

Labelled CRBC were incubated for varying times in the presence of spleen cells of the C57BL/6 mice which were injected intraperitoneally with 1×10^8 CRBC 4 days before (Fig. 1). A very low degree of ^{51}Cr-release was detected after incubation for 3 h.

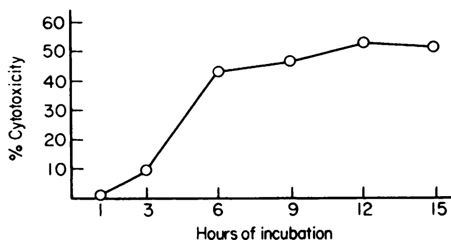

Figure 1. Time course of ^{51}Cr-release from labelled CRBC in the presence of spleen cells of C57BL/6 mice immunized intraperitoneally with 1×10^8 CRBC 4 days before.

The specific release reached 43% after incubation for 6 h and increased scarcely thereafter. Thus, 6 h was chosen as the standard incubation time throughout this study.

Strain differences in cytotoxicity to CRBC

C57BL/6, C3H/He, AKR, SL and DDD mice were injected intraperitoneally with 1×10^8 CRBC. Cytotoxicity of spleen cells was assessed at 4, 8 and 14 days after immunization (Fig. 2). A high degree

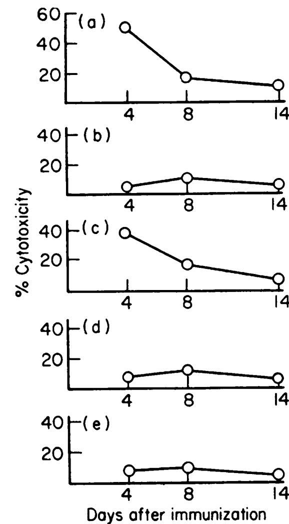

Figure 2. Strain differences in the induction of cytotoxicity of spleen cells against CRBC after intraperitoneal immunization with 1×10^8 CRBC. Cytotoxicity is expressed as percent release of ^{51}Cr. (a) C57BL/6; (b) C3H/He; (c) AKR; (d) SL; (e) DDD.

of cytotoxicity was detected in C57BL/6 and AKR mice on day 4. Very low or negligible degrees of cytotoxicity were detected in SL, DDD and C3H/He mice on day 4. Cytotoxicity became scarcely detectable on day 8 and 14 in all the strains. Spleen cells of non-immunized mice of any strain did not exhibit a detectable degree of cytotoxicity.

Precise time course studies on the induction of cytotoxicity were carried out with C57BL/6 and C3H/He mice. Cytotoxicity of spleen cells was assessed on days 1, 2, 3, 4, 5, 6, 7 and 8 after immunization on day 0 (Fig. 3). In C57BL/6 mice, a low degree of cytotoxicity was detected on day 3. Cytotoxicity reached the peak on days 4 and 5 and declined thereafter to reach near the control level by day 8. On the other hand, a moderate

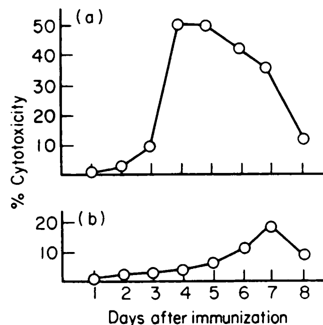

Figure 3. Precise time course study on cytotoxicity of spleen cells against CRBC after intraperitoneal immunization with 1×10^8 CRBC in C57BL/6 and C3H/He mice. Cytotoxicity is expressed as percent release of ^{51}Cr. (a) C57BL/6; (b) C3H/He.

degree of cytotoxicity was detected only on day 7 in C3H/He mice.

Cytotoxicity was detected in both of C57BL/6 (cytotoxicity 59·5%) and C3H/He mice (cytotoxicity 49·4%) 4 days after an intraperitoneal booster in the animals which had been immunized intraperitoneally 14 days before the booster. High titres of antibody were detected in the sera of these mice.

Dose dependence of the induction of cytotoxicity

Varying numbers of CRBC in saline were injected intraperitoneally into C57BL/6 mice and cytotoxicity of spleen cells was assessed 4 days later (Fig. 4). Cytotoxicity was not induced with 1×10^6 CRBC and was raised weakly with 1×10^7 CRBC. A high degree of cytotoxicity was induced with 1×10^8 CRBC. Immunization with 1×10^9 CRBC induced a higher degree of cytotoxicity than with 1×10^8. The dose of 1×10^8 was chosen as a standard dose for immunization throughout this study.

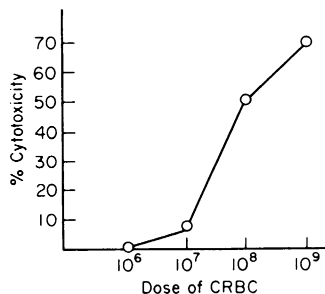

Figure 4. Dose-dependence of cytotoxicite of spleen cells against CRBC in C57BL/6 mice. Cytotoxicity was assessed 4 days after intraperitoneal immunization, and is expressed as percent release of ^{51}Cr.

Table 1. Effects of treatment with anti-θ serum or anti-IgG serum and complement (C) on the cytotoxicity of C57BL/6 spleen cells against CRBC or C3H/He spleen cells against allogeneic tumour cells

Treatment of spleen cells	Cytotoxicity* CRBC day 4†	Cytotoxicity* CRBC day 7	Cytotoxicity* EL-4 day 7
None	32·4	16·8	43·5
Normal AKR serum + C	49·4	18·2	57·3
Anti-θ serum + C	13·0	2·6	20·8
Anti-IgG serum + C	7·0	1·6	66·9

*Cytotoxicity is expressed as percent release of ^{51}Cr.

† Days after an intraperitoneal injection with 1×10^8 CRBC or a subcutaneous injection with 1×10^6 EL-4 cells.

Dependence of cytotoxicity on θ-positive and IgG-positive lymphocytes

C57BL/6 mice were injected intraperitoneally with 1×10^8 CRBC and cytotoxicity of spleen cells, cervical lymph node cells, mesenteric lymph node cells and peritoneal cells was assessed 4 days later. High degrees of cytotoxicity were detected in spleen cells (44·1%) and peritoneal cells (35·0%). The degrees of cytotoxicity were low in cervical lymph node cells (19·1%) and mesenteric lymph node cells (9·0%).

C57BL/6 mice were injected intraperitoneally with 1×10^8 CRBC and cytotoxicity of spleen cells was assessed after treatment with anti-θ serum or anti-IgG serum and complement on days 4 and 7 (Table 1). C3H/He mice were inoculated subcutaneously with 1×10^6 EL-4 cells and cytotoxicity of spleen cells against EL-4 cells was assessed on day 7 as a control. Cytotoxicity against allogeneic EL-4 cells was diminished to a substantial extent by treatment with anti-θ serum and complement, but not by treatment with anti-IgG serum and complement. On the other hand, cytotoxicity against CRBC was diminished remarkably not only by treatment with anti-θ serum and complement, but also by treatment with anti-IgG serum and complement.

AKR mice were injected intraperitoneally with 1×10^8 CRBC and the effects of treatment with anti-θ serum and anti-IgG serum on the cytotoxicity of spleen cells were examined 4 days later. Cytotoxicity was diminished by both treatment with anti-θ serum or anti-IgG serum and complement.

Dependence of cytotoxicity on glass-nonadherent and Nylon wool column-adherent cells

C57BL/6 mice were immunized intraperitoneally with 1×10^8 CRBC and the cytotoxicity of spleen cells was assessed 4 days later. Cytotoxicity was found to reside in the population which did not adhere to the glass surface of a culture bottle (Table 2). However, cytotoxicity was not found in the population which passed through a Nylon wool column (Table 3). Cytotoxicity was found to reside in the population which adhered to a Nylon wool column and was destroyed by treatment with anti-θ serum or anti-IgG serum and complement.

Table 2. Dependence of cytotoxicity on glass-non-adherent cells in C57BL/6 mice immunized intraperitoneally with 1×10^8 CRBC 4 days before

Spleen cells	Cytotoxicity*
Non-immune cells	−4·3
Whole immune cells	47·5
Glass-nonadherent immune cells	41·8

* Cytotoxicity is expressed as percent release of ^{51}Cr.

Lymphocyte subpopulations after various treatments

Total number of normal C57BL/6 spleen cells was reduced to 78, 60, 98 and 94% of a non-treated specimen by treatment with anti-θ serum and complement, anti-IgG serum and complement, normal AKR serum and complement, and complement alone, respectively. The number was reduced to 41% by a treatment with both anti-θ serum and anti-IgG serum and complement. When spleen cells were obtained 5 days after intraperitoneal immunization with CRBC, similar degrees of reduction were obtained after such treatments. When such immune spleen cells were passed through a nylon wool column, approximately 20% and 70% of starting material was harvested as non-adherent and adherent cells, respectively. The number of harvested adherent cells was reduced to 45, 21 and 73% by treatment with anti-θ serum and complement, anti-IgG serum and complement, and normal AKR serum and complement, respectively.

Table 3. Adherence of θ-positive cytotoxic lymphocytes to nylon wool column

Spleen cells*	Cytotoxicity†
Non-treated whole cells	20·8
Non-treated nonadherent cells	−0·5
Adherent cells treated with normal AKR serum and C‡	20·3
Adherent cells treated with anti-θ serum and C	3·2
Adherent cells treated with anti-IgG serum and C	8·3

* Spleen cells of C57BL/6 mice were obtained 4 days after an intraperitoneal injection with 1×10^8 CRBC.

† Cytotoxicity is expressed as percent release of ^{51}Cr.

‡ Complement.

θ- and IgG-antigens on the cell surface were examined by an indirect and a direct immunofluorescence method. In a non-treated population of normal spleen cells, 28 and 20% were stained as B and T cells, respectively. When the cells were pretreated with anti-θ serum and complement, 33 and 6% of surviving cells were stained as B and T cells, respectively. When the cells were pretreated with anti-IgG serum and complement, 4 and 59% of surviving cells were stained as B and T cells, respectively. Similar results were obtained with immune spleen cells.

Effector cells in ADCC against CRBC

C57BL/6 mice were injected intraperitoneally with 1×10^8 CRBC and given an intraperitoneal booster immunization 14 days later. Sera were obtained 4 and 14 days after primary immunization and 4 days after the booster. *In vitro* experiments for ADCC were carried out with normal spleen cells of C57BL/6 mice. ^{51}Cr-release in ADCC was very low in the presence of 4 day-serum (−0·1 ~ 9·3%), while it was high in the presence of 14 day-serum (30·3 ~ 34·2%). ^{51}Cr-release was very high in the presence of booster-serum (43·1 ~ 49·3%). ^{51}Cr-release in ADCC was diminished by treatment of spleen cells with anti-IgG serum and complement, but not by that with anti-θ serum and complement in the presence of 14 day-serum or booster-serum (Table 4).

Antibody production and delayed hypersensitivity

C57BL/6 and C3H/He mice were injected intraperitoneally with 1×10^8 CRBC. Antibody was produced efficiently in both the strains during the observation period of 15 days (Fig. 5). 2-ME resistant antibody became detectable on day 5 and the titres on day 7 consisted largely of 2-ME resistant antibody.

Table 4. Non-sensitivity of effector cells in ADCC to treatment with anti-θ serum and complement (C)

Treatment of normal spleen cells of C57BL/6 mice	Cytotoxicity* in the presence of	
	14-day-serum†	booster-serum‡
None	24·5	47·4
Normal AKR serum and C	21·7	44·8
Anti-θ serum and C	16·2	31·7
Anti-IgG serum and C	1·7	15·5
Control (antibody only)	−4·5	1·5

* Cytotoxicity is expressed as percent release of ^{51}Cr.

† Serum of C57BL/6 mice was harvested 14 days after primary intraperitoneal immunization.

‡ Serum of C57BL/6 mice was harvested 4 days after secondary intraperitoneal immunization.

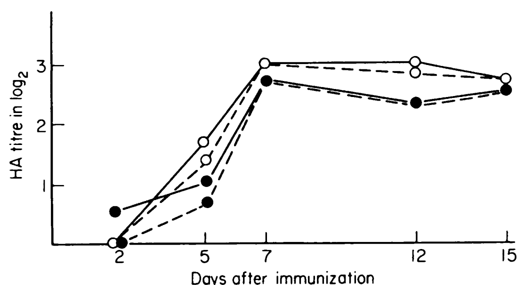

Figure 5. Antibody production after intraperitoneal immunization with 1×10^8 CRBC in C57BL/6 and C3H/He mice. ●——● Total HA titre in C57BL/6 mice; (●– – –●) 2-ME resistant HA titre in C57BL/6 mice; (○——○) Total HA titre in C3H/He mice; (○– – –○) 2-Me resistant HA titre in C3H/He mice.

C57BL/6 and C3H/He mice were injected intraperitoneally with 1×10^8 CRBC and PFC in spleen were counted on day 4. Numbers of PFC per 10^6 spleen cells in these strains were within the same range (C57BL/6: 156·8, C3H/He: 104·6). PFC adhered to Nylon wool column. PFC in whole spleen cell population and Nylon wool column-adherent cell population were resistant to treatment with anti-θ serum and complement (Table 5).

50 μl of saline containing 1×10^8 CRBC were injected into the right hind footpad on day 4, 5 or 6 after intraperitoneal immunization and footpad swelling was read 24 and 48 h after the challenge (Fig. 6). Positive reactions were detected in both the strains, although slightly better reactions were detected in C3H/He mice.

Effect of route of immunization

Efficiency of immunization through the footpad or vein was studied in C57BL/6 and C3H/He mice which received 1×10^8 CRBC. Cytotoxicity of spleen cells, antibody production and delayed footpad reaction were assessed 4 and 8 days after the immunization (Table 6). Immunization through the footpad was not effective in the induction of cytotoxicity and antibody production in both strains, whereas it evoked a high degree of delayed footpad reaction. Cytotoxicity was detected in both strains after intravenous immunization, although the level was higher in C57BL/6 mice than in C3H/He mice. Antibody production was similar in both strains. On the other hand, footpad reactions were weak in both strains after intravenous immunization, although a slightly better reaction was detected in C3H/He mice.

Cytotoxicity was depleted completely by treatment with anti-θ serum and complement from spleen cells of C57BL/6 mice which had been intravenously immunized with 1×10^8 CRBC 4 days before.

Table 5. Non-sensitivity of PFC to a treatment with anti-θ serum and complement (C)

Source of spleen cells*	Treatment of spleen cells	PFC/10^6 spleen cells
Whole cells	None	213
	Normal AKR serum + C	171
	Anti-θ serum + C	140
Nylon wool column-adherent cells	None	270
	Normal AKR serum + C	174
	Anti-θ serum + C	197

* C57BL/6 mice were injected intraperitoneally with 1×10^8 CRBC 4 days before.

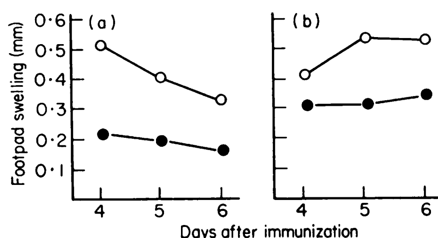

Figure 6. Delayed footpad reaction after intraperitoneal immunization with 1×10^8 CRBC in C57BL/6 and C3H/He mice. (○——○) Footpad swelling 24 h after challenge; (●——●) Footpad swelling 48 h after challenge. (a) C57BL/6; (b) C3H/He.

Effect of oil adjuvants on the induction of cytotoxicity

C57BL/6 mice were injected subcutaneously with CRBC in FCA or FIA and cytotoxicity of spleen cells were assessed 4 and 7 days later. Cytotoxicity was not increased after such treatment.

Non-cross-reactivity between chicken erythrocytes and kidney cells

C57BL/6 and C3H/He mice were injected intraperitoneally with 1×10^8 CRBC or 1×10^7 kidney cells and the cytotoxicity of spleen cells against labelled CRBC and kidney cells was assessed 4 days later. When CRBC were used as an immunogen and a target, a high degree of cytotoxicity was detected only in C57BL/6 mice. When kidney cells were used as an immunogen and a target, a high degree of cytotoxicity was detected in both C57BL/6 and C3H/He mice. Cross-reactivity was not detected between CRBC and kidney cells in terms of an immunogen and a target.

DISCUSSION

CRBC have been used widely as target cell in antibody-dependent cell-mediated cytotoxicity (ADCC), since the cytotoxic effect on CRBC is assessed readily by an assay of ^{51}Cr-release (Perlman & Perlman, 1970). However, there is some controversy with respect to the induction of cytotoxicity in the lymphoid cells after active immunization and the cell types responsible for such cytotoxicity. Loewi & Temple (1972) reported that cytotoxic activity was detectable in spleen or peritoneal exudate cells after an intravenous injection of CRBC in guinea-pigs. They suggested that cytotoxicity was associated with macrophages when peritoneal exudate cells served as the source of effector cells. Dennert & Lennox (1973) reported the induction of cytotoxicity in spleen cells after an intravenous injection with CRBC in mice. They suggested that it was antibody-dependent cytotoxicity mediated by phagocytic cells. In our study, CRBC induced cytotoxicity in spleen cells of mice after intraperitoneal or intravenous immunization. The cytotoxicity of spleen cells was shown to reside in a population of glass-nonadherent cells and to be diminished by a treatment with anti-θ serum and complement. These results suggest that the cytotoxicity against CRBC in our study depends upon a direct cytotoxic activity of θ-positive cells, presumably T cells, at least with respect to spleen cells. Discrepancies among the results of Loewi & Temple (1972), those of Dennert & Lennox (1973) and ours may be ascribed to variations in experimental design. Loewi & Temple (1972) assessed cytotoxicity at 1, 2, or 3 weeks after immunization in guinea-pigs. Dennert & Lennox (1973) detected cytotoxicity mostly at 2 weeks after immunization or later. In

Table 6. Effect of immunizing route on the induction of immune responses against CRBC in C57BL/6 and C3H/He mice

Route of immunization (CRBC 1×10^8)	Strain of mice	Cytotoxicity* on day		HA titres ($\log_2$) on day		Footpad reaction on day	
		4	8	4	8	4	8
Footpad	C57BL/6	−6·8	−3·7	<1·0	1·0	0·96	0·47
	C3H/He	−3·4	−4·1	<1·0	1·0	0·55	0·57
Intravenous	C57BL/6	44·0	50·0	2·5	3·7	0·19	0·11
	C3H/He	17·6	28·2	2·7	3·3	0·35	0·31

* Cytotoxicity is expressed as percent release of ^{51}Cr.

our experiment, direct cytotoxicity of spleen cells was detectable from day 3 to 7 after immunization. The timing of assessment may be one of the important factors responsible for such discrepancies. The source of effector cells and the species or strain of host may be also important factors.

The cytotoxicity of spleen cells detected at early stages was found to be attributable to θ-positive cells. However, these cytotoxic lymphocytes showed some features by which they were distinguished from typical cytotoxic T lymphocytes against allogeneic cells. Cytotoxic T lymphocytes against allogeneic tumour cells were not eliminated by a treatment with anti-IgG serum and complement, while cytotoxic θ-positive cells against CRBC were destroyed by such a treatment. Cytotoxic T lymphocytes against allogeneic cells were found to pass through a Nylon wool column (Kiessling, Petranyi, Karre, Jondal, Tracey & Wigzell, 1976). Cytotoxic activity to CRBC was shown to reside in a cell population adherent to a Nylon wool column. Cytotoxic lymphocytes eluted from the column were destroyed by treatment with anti-θ serum or anti-IgG serum and complement. Thus, cytotoxicity against CRBC observed at early stages after immunization appears to reside in a cell population which is θ-positive, IgG-positive, Nylon wool-adherent and glass-nonadherent. IgG on these θ-positive cells may be derived from IgG-secreting plasma cells. However, the possibility exists that cytotoxic T lymphocytes against CRBC express surface immunoglobulins to a greater extent than those against allogeneic cells. The origin and significance of IgG on the surface of θ-positive cells will be analysed in further studies. The T cell nature of cytotoxicity against CRBC may be supported also by the fact that cytotoxic activity can be induced in lethally irradiated, thymus cell-transferred mice. Any immune response against CRBC was not induced in lethally irradiated mice in the absence of thymus cell-transfer. Detail of these results will be reported in a following paper.

The second possible effector cell type in cytotoxicity against CRBC may be phagocytes which can engulf CRBC efficiently in the presence of antibody or work as effector cells of ADCC also in the presence of antibody. Cytotoxic activity of spleen cells obtained at 4 days after immunization was not attributed to phagocytes, since their cytotoxicity was not diminished after the removal of glass-adherent cells from the whole spleen cells. However, the cytotoxicity of peritoneal cells was not diminished by treatment with anti-θ serum and complement, but was reduced to a great extent by the removal of glass-adherent cells. Therefore, cytotoxic activity may reside in a population with phagocytic activity, even when peritoneal cells are obtained at early stages after primary immunization.

The third possible effector mechanism may be a system of ADCC, which is mediated by B lymphocytes, as suggested by Loewi & Temple (1972) and Dennert & Lennox (1973). In our system, ADCC was not detected in the presence of sera obtained 4 days after primary immunization, at which time a high degree of cytotoxicity was detected in spleen cells. On the other hand, a high degree of ADCC was detected in the presence of sera obtained 14 days after primary immunization at which time direct cytotoxicity was scarcely detected in spleen cells. Antibody production against CRBC was induced to almost the same extent in both C57BL/6 and C3H/He mice, although a direct cytotoxicity was detected only in C57BL/6 mice. Therefore, the direct cytotoxicity of spleen cells observed after primary immunization may not be ascribed to ADCC which may be evoked in the presence of antibody produced *in vitro* by plasma cells or antibody produced *in vivo* by plasma cells and carried by B lymphocytes. However, ADCC may participate in cytotoxicity against CRBC under several conditions. Cytotoxicity after booster immunization may be ascribed to ADCC, since cytotoxic activity resistant to treatment with anti-θ serum and complement could be detected in some of various experimental designs. Experiments will be conducted to determine the factors by which the development of ADCC in immune lymphoid cells is controlled.

The fourth possible effector cell type in direct cytotoxicity may be PFC which are producing antibody molecules to CRBC, since the time course in the appearance of PFC was correlated with that of a direct cytotoxicity. In our system, PFC were not depleted selectively by a treatment with anti-θ serum and complement, while direct cytotoxicity on the same day was depleted by such treatment. Almost the same number of PFC were detected in the spleen cells of C57BL/6 and C3H/He mice, while direct cytotoxicity in such spleen cells was detected only in C57BL/6 mice. Therefore, PFC may be excluded as effector cells of direct cytotoxicity.

The fifth possible effector cell type in direct cytotoxicity may be sensitized lymphocytes responsible

for delayed hypersensitivity. Tubercle bacilli-sensitized lymphocytes were found to kill bystander target cells in the presence of PPD in culture medium (Ruddle & Waksman, 1967). In our system, delayed footpad reactions of a comparable degree were elicited in C57BL/6 and C3H/He mice after intraperitoneal immunization with CRBC, while a direct cytotoxicity was detected only in C57BL/6 mice. Although delayed footpad reactions of a very high degree were elicited in mice immunized with CRBC in FIA or FCA in preliminary experiments, a direct cytotoxicity was not detected in their spleen cells. Thus, sensitized lymphocytes responsible for delayed hypersensitivity may be excluded as effector cells in a direct cytotoxicity.

The route of immunization is a very important factor in the induction of immune responses against CRBC in mice. The intraperitoneal route was effective in the induction of cytotoxicity, delayed hypersensitivity and antibody production. Immunization via the footpad was shown to be very effective in the induction of delayed hypersensitivity as demonstrated by footpad swelling, but not in the induction of cytotoxicity and antibody production. The intravenous route was effective in the induction of cytotoxicity and antibody production, but not in the induction of delayed footpad reaction. These results may support the concept that various immune responses against CRBC as described above depend upon distinct subpopulations of differentiated T cells.

Cytotoxic activity was greater in spleen cells than in cervical or mesenteric lymph node cells after intraperitoneal immunization with CRBC. The spleens may be comparable to the regional lymph nodes after skin-grafting, when CRBC are injected intraperitoneally. Therefore, the greater a direct cytotoxicity of spleen cells seems to be reasonable. The lower cytotoxicity of mesenteric lymph node cells cannot be understood at the present time.

Genetic controls on T cell-mediated cytotoxicity have been reported only in a few systems. Vachek & Kölsch (1974) have described a genetically controlled low responsiveness against DBA/2 mastocytoma P-815 in the DBA/1 host. In their experiments, low responsiveness of this strain was inherited as a recessive trait. Schmitt-Verhulst & Shearer (1975) have reported that spleen cells from two C57BL/6 congeneic strains sharing common 1-C, S and D regions, but differing at K, 1-A and 1-B regions generate different levels of lytic response to the shared modified H-2D products upon sensitization with autologous TNP-modified cells. In their experiments, responsiveness was expressed as a dominant trait in the F_1 mice. The pattern of inheritance of cytotoxicity to CRBC will be analysed further in this series.

C57BL/6 and C3H/He mice responded well with respect to antibody production and development of delayed hypersensitivity to CRBC. In many cases, an immune response gene regulates all functions of T cells such as helper function in antibody production and development of delayed hypersensitivity as reported by many investigators (Nomoto, Makidono & Takeya, 1972; Nomoto, Yamada, Muraoka & Takeya, 1973). The dissociation between cytotoxicity and other immune responses may be explained by the following mechanisms. (1) Cytotoxicity and other immune responses may be induced against distinct antigenic determinants. Thus, cytotoxicity may be regulated by an immune response gene different from those for antigenic determinants in antibody production and delayed hypersensitivity. (2) Cytotoxicity and other immune responses may be induced against the same antigenic determinant. Thus, C57BL/6 and C3H/He mice may recognize the antigen with the same efficiency. However, the generation of cytotoxic lymphocytes may require the participation of another gene.

CRBC and kidney cells induced cytotoxicity against the immunizing cells themselves. No cross-reactivity exists between both types of cells in the induction and expression of cytotoxicity. Kidney cells induced cytotoxicity of a comparable degree against kidney cells in both C57BL/6 and C3H/He mice, while CRBC induced cytotoxicity against CRBC more efficiently in C57BL/6 than in C3H/He mice. Thus, cytotoxicity against CRBC and kidney cells may be directed towards different antigens.

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