XI. Suppression of Primary IgM and IgG Plaque-Forming Cell Responses In Vitro by Alloantisera Against Leukocyte Alloantigens*

BY CARL W. PIERCE,† JUDITH A. KAPP, SUSAN M. SOLLIDAY,§ MARTIN E. DORF, AND BARUJ BENACERRAF

(From the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115)

Interactions among macrophages (Mφ), T cells, and precursors of antibody-producing (B) cells in the presence of antigen, mediated by direct cell-to-cell contact(s) and/or by soluble factors elaborated by one or more of the cells, are essential for development of optimal plaque-forming cell (PFC) responses to "thymus-dependent" antigens such as sheep erythrocytes (SRBC) and random terpolymer of L-glutamic acid−L-alanine−L-tyrosine (GAT) by mouse spleen cells in vitro (1–4). Recent experiments have defined some of the genetic requirements for successful physiologic interactions among antigen and these cells. First, Mφ and lymphoid cells (T and B cells) need not be syngeneic for successful generation of PFC responses; allogeneic Mφ support the development of PFC responses by lymphoid cells which are comparable in magnitude to responses which develop in the presence of syngeneic Mφ (4–6). Thus no known genetic restrictions appear to govern the interactions of Mφ with T cells and B cells in the development of antibody responses in the mouse. Second, physiologic cooperation between T and B cells requires that the T cells be syngeneic or semisyngeneic with B cells at the H-2 complex (7, reviewed in reference 8). It has been shown that identity of certain membrane molecules encoded by the H-2 complex is required for antigen-activated T cells to be able to supply the appropriate "second" stimulus required to trigger antigen-activated B cells into cycles of cell division and differentiation into mature antibody-producing cells. These membrane molecules may be the H-2 antigens themselves or other molecules coded for by the K end of the H-2 complex (8). In addition to the mechanism of T-cell-B-cell interaction requiring physical contact between crucial membrane molecules on T and B cells, humoral factors produced by activated T cells may also act on the membrane molecules on B cells to

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§ Present address: Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77025.

Abbreviations used in this paper: B cells, precursors of antibody-producing cells; GAT, random terpolymer of L-glutamic acid−L-alanine−L-tyrosine; HBSS, Hanks' balanced salt solution lacking sodium bicarbonate; Ir gene, H-2-linked immune response gene; Mφ, macrophages; PFC, plaque-forming cell(s).
provide the appropriate second stimulus (8, 9). Thus, gene products of the H-2 complex located on the membranes of the T and B cells play a crucial role in the successful generation of antibody responses.

We have used the suppressive effects of alloantisera against leukocyte alloantigens on PFC responses in vitro as a model system to investigate which of the required interactions among antigen, MΦ, T cells, and B cells may be mediated by membrane molecules coded for by the H-2 complex. In this communication we shall define some of the important parameters in suppression of PFC responses in vitro by antisera against leukocyte alloantigens and the cellular sites of action of suppressive alloantisera.

Materials and Methods

**Mice.** C57BL/6 (H-2b), SJL/J (H-2d), A/J (H-2h), DBA/2J (H-2b), C3H/HeJ (H-2k), and B10.D2n (H-2s) male mice were purchased from Jackson Laboratories, Bar Harbor, Maine. A.Sw (H-2k) male mice were purchased from Dr. G. Haughton, University of North Carolina, Chapel Hill, N.C.; B10.S (H-2s) male mice were produced in our animal facilities from breeding stock from Dr. D. Shreffler, University of Michigan, Ann Arbor, Mich. The F1 hybrid mice of (C57BL/6 × SJL) (H-2b), (B10.A × B10.S) (H-2a), and (C57BL/6 × B10.S) (H-2a) matings were produced in our animal facilities. Mice used in these studies were 3- to 6-mo old and were maintained on acidified-chlorinated water and laboratory chow ad libitum.

**Antigens.** SRBC (Grand Island Biological Co., Grand Island, N. Y.) were prepared for use as antigen in cultures or as indicator cells in the hemolytic plaque assay as described previously (10). A sterile stock solution of 10 mg GAT/ml (Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill.) with an average mol wt of 32,000 was prepared and diluted for addition to cultures as antigen or for preparation of GAT-SRBC for indicator cells in the hemolytic plaque assay as described previously (11).

**Alloantisera.** Four polyspecific alloantisera, DBA/2 anti-C57BL/6; C3H/He anti-SJL; SJL anti-C57BL/6; and C57BL/6 anti-SJL, were prepared in our laboratory by injecting recipient mice intraperitoneally (i.p.) at 2-wk intervals with 25 × 10⁶ spleen cells from donor mice. 7 days after the eighth and subsequent injections, recipient mice were bled and the serum was separated and stored at −20°C. Alloantisera with activity against antigens coded for by defined regions of the H-2 complex were gifts from Dr. D. Shreffler and Dr. G. Snell (Jackson Laboratories). The strain combinations used in the production of these sera and the specificities against which the sera are reactive are detailed in Table II. All alloantisera were decomplemented by heating at 56°C for 30 min, absorbed three times at 4°C with SRBC equivalent to 20% of the volume of serum, and centrifuged at 40,000 rpm for 2 h at 0°C in a SW 50.1 rotor in a Beckman Model L3-50 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). The sera were sterilized by membrane filtration and stored at −20°C in 0.1-ml samples. The alloantisera were diluted as indicated in Hanks’ balanced salt solution lacking sodium bicarbonate (HBSS) and 50 μl were added to cultures without complement. Cytotoxicity titers of the four polyspecific alloantisera against ¹⁴Cr-labeled donor strain spleen cells were comparable. The DBA anti-C57BL/6 serum had negligible activity against SJL spleen cells; the C3H anti-SJL serum had negligible activity against C57BL/6 spleen cells. The alloantisera against antigens coded for by defined regions of the H-2 complex were diluted so that cytotoxicity titers were comparable when tested against spleen cells from the appropriate strains of mice. The one exception was the A.TL anti-A.TH serum which was from an early bleeding in the immunization schedule and had no detectable cytotoxic activity against SJL, B10.S, or A.Sw spleen cells. However, since this serum had activity against A/J thymocytes due to anti-TL antibodies, it was exhaustively absorbed with A/J thymocytes before centrifugation and filtration.

**Spleen Cell Cultures and PFC Assays.** Dispersed, single spleen cells or separated splenic lymphoid cells at 10 × 10⁶ cells/ml in completely supplemented Eagle’s minimal essential medium
containing 10% fetal bovine serum (lot HT3212, Reheis Chemical Co., Kankakee, Ill.) were incubated with or without 10⁶ splenic adherent cells or 7 × 10⁴ peritoneal exudate MΦ and antigens under modified Mishell-Dutton conditions (10). Cultures were stimulated with 10⁷ SRBC, 10 μg GAT, or GAT bound to MΦ and alloantisera were added according to the experimental protocol. PFC responses to SRBC or GAT were measured after 5-days incubation using the modifications of the Jerne hemolytic plaque assay described previously (10, 11). Data are expressed as PFC per culture or as the mean of the percent of control responses, calculated as follows: (PFC/culture:alloantiserum treated + PFC/culture:untreated) × 100 from three to five experiments. The range of individual values around the mean did not exceed 15%.

Preparation of Splenic Lymphoid Cells and Splenic and Peritoneal Exudate MΦ. Spleen cell suspensions were separated into splenic MΦ and lymphoid cell populations by serial passage over plastic Petri dishes (1). Depending on the experimental protocol, the separated populations were either recombined in the presence of antigen and alloantiserum immediately or incubated with alloantiserum for 24 h, washed with HBSS, and then recombined in the presence of antigen. Peritoneal exudate MΦ were collected from mice injected i.p. 3 days before with 1-ml sterile 10% proteose peptone broth (6, 12).

Preparation of MΦ-Bound GAT. The preparation of GAT-MΦ has been described previously (12). Briefly, 4 × 10⁶ peritoneal exudate MΦ in 2 ml HBSS were reacted with 200 μg GAT containing 2 μg[^12]I-GAT for 30 min at 4°C. In some experiments a portion of the macrophages were reacted with alloantiserum (final dilution 1/100) at 4°C for 30 min before the addition of GAT or MΦ were reacted with alloantiserum but not with GAT. All MΦ preparations were held at 4°C for an equal period of time.

The MΦ were then washed three times with 50 ml HBSS and resuspended in HBSS at 7 × 10⁴ MΦ/ml. The radioactivity in a portion of each MΦ preparation was counted in a Packard autogamma scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.) and the amount of GAT bound to 7 × 10⁴ MΦ was calculated. 7 × 10⁴ MΦ in 0.1 ml HBSS were added to cultures of 10 × 10⁴ splenic lymphoid cells according to the experimental protocol.

Cell Counts. Nucleated cells were enumerated with a Model B Coulter Counter (Coulter Electronics, Inc., Fine Particle Div., Hialeah, Fla.). Cell viability was determined by trypan blue exclusion with a Cytograf Model 6300A (Bio/physics Systems, Inc., Mahopac, N. Y.).

Results

Effects of Polyspecific Alloantisera against Leukocyte Alloantigens on PFC Responses In Vitro. Cultures of spleen cells from normal C57BL/6 and DBA/2 mice were stimulated with SRBC; DBA/2 anti-C57BL/6 serum was added at various final dilutions at culture initiation. Day 5 IgM and IgG PFC responses in alloantiserum-treated cultures are expressed as the percent of responses in control cultures to which no alloantiserum was added (Fig. 1). The DBA/2 anti-C57BL/6 serum suppressed both IgM and IgG PFC responses by C57BL/6 spleen cells 75% or more at final dilutions from 1/20 to 1/160. As the serum was diluted further, the suppressive effect disappeared; significant enhancement of PFC responses was not observed with any serum dilution. The alloantiserum did not significantly affect IgM and IgG (data not shown) PFC responses by DBA/2 spleen cells. Viable cell recovery in all cultures was approximately 2 × 10⁴ cells/culture. Identical titration experiments using the other three polyspecific alloantisera showed that the alloantisera had no significant effect on PFC responses by spleen cells from the strain of mice in which the serum was prepared. Results completely analogous with those in Fig. 1 were obtained when alloantisera were added to cultures of spleen cells from the mouse strains against which the alloantiserum was directed.
Portions of each of the four polyspecific alloantisera were absorbed three times at 4°C for 30 min with ethylchloroformate insolubilized (13) Ig (1 mg Ig/ml serum/absorption) or spleen cells (1 ml cells/ml serum/absorption) from the mouse strain in which the serum was prepared or against which the serum was directed. The sera were added to cultures of spleen cells from mouse strains against which the alloantiserum was directed at a final dilution of 1/100 at initiation. The results were identical for all four polyspecific alloantisera; only absorption with spleen cells from the mouse strain against which the serum was directed removed the suppressive activity from the alloantiserum.

**Effects of Alloantiserum Added after Culture Initiation on PFC Responses In Vitro.** Cultures of spleen cells from strains of mice against which the four polyspecific alloantisera were directed were established and the alloantiserum was added at a final dilution of 1/100 at initiation or at 24-h intervals thereafter. IgM and IgG PFC responses were determined at 120 h. Results of all these experiments were comparable; data using DBA/2 anti-C57BL/6 serum in cultures of C57BL/6 spleen cells were exemplary (Fig. 2). When the alloantiserum was added at culture initiation, IgM and IgG PFC responses were suppressed 80% or more. As the interval after culture initiation when the alloantiserum was added increased, suppression of the PFC responses progressively decreased. Alloantiserum added at 72 h had no effect on IgM PFC responses, but IgG responses were still suppressed approximately 40%; alloantiserum added at 96 h had no effect on IgM or IgG PFC responses. In other experiments, comparison of the kinetics of IgM and IgG PFC responses in control cultures and cultures to which alloantiserum was added at initiation clearly demonstrated that the 80% or greater suppression of 5-day PFC responses was due to a failure to successfully initiate the PFC responses.

**Effect of Removing Noncell-Bound Alloantibodies after Various Intervals of Incubation on PFC Responses In Vitro.** Cultures of spleen cells from strains of
mice against which the four polyspecific alloantisera were directed were established and the alloantiserum was added to one-half of the cultures at a final dilution of 1/25 at culture initiation. At various intervals control and alloantiserum-treated cultures were harvested, spleen cells were washed twice with HBSS, resuspended in fresh medium at 10 x 10^6 cells/ml, and returned to original culture dishes. Cells adherent to these dishes had been washed twice with HBSS. PFC assays were performed on day 5. The data from experiments using the four polyspecific antisera were entirely comparable; results with DBA/2 anti-C57BL/6 serum are shown (Fig. 3). PFC responses in cultures exposed to alloantiserum for 48 h were suppressed to the same degree as responses in cultures exposed to alloantiserum for 120 h. Exposure to alloantiserum for 24 h resulted in slightly less suppression than exposure for 48 h; a 6-h exposure to alloantiserum had no significant effect on PFC responses.

In other experiments not shown here, we examined whether spleen cells were rendered totally incapable of developing a PFC response after exposure to alloantiserum for 48 h. The results clearly indicated that the alloantiserum-mediated suppression of PFC responses was not the result of an irreversible inactivation of responding spleen cells. Rather, spleen cells after removal of alloantiserum at 48 h developed significantly greater PFC responses than spleen cells continuously exposed to alloantiserum when the incubation period was extended to 7 days.

**Effect of Polyspecific Alloantisera on PFC Responses In Vitro by Spleen Cells from Various Mouse Strains.** The effects of the four polyspecific alloantisera on PFC responses by spleen cells from several mouse strains were examined to determine whether suppression was mediated by antibodies against antigens encoded by the H-2 complex only or whether antibodies to non-H-2 antigens were also involved and whether antibodies to antigens of one H-2 haplotype in an F1 hybrid were capable of mediating suppression (Table I). First, each serum
suppressed IgM and IgG PFC responses by spleen cells from the mouse strains against which it was directed; none suppressed responses by A/J spleen cells even though both anti-SJL sera contained antibodies reactive with A/J cells by hemagglutination. The two sera against SJL antigens suppressed PFC responses by A.Sw spleen cells (H-2a haplotype on the A strain background) indicating that antibody against H-2 antigens can effectively mediate the suppression. However, all four antisera suppressed responses by B10.S spleen cells (H-2b haplotype on the C57BL/10 background) and the DBA/2 anti-C57BL/6 serum suppressed PFC responses by B10.D2n spleen cells (H-2d haplotype on the C57BL/10 background) (data not shown). These data indicate that antibody to non-H-2 as well as H-2 antigens can mediate the suppression. This is especially evident in the case of the SJL anti-C57BL/6 serum which should be reactive against only the non-H-2 antigens on B10.S spleen cells and the C57BL/6 anti-SJL serum which should be primarily reactive with the H-2b antigens on B10.S spleen cells. Suppression of responses by (C57BL/6 × SJL)F1 spleen cells by all four alloantisera demonstrated that antibody against the antigens contributed by either parent in the F1 hybrid can effectively suppress PFC responses. The last two F1 hybrid strains (C57BL/6 × B10.S)F1 and (B10.A × B10.S)F1 reiterate all the previous points about suppression of PFC responses by alloantisera. That is, antibody against H-2 and non-H-2 antigens can mediate suppression and antibody against antigens of only one parent on F1 hybrid spleen cells is sufficient to suppress IgM and IgG PFC responses.

Effect of Alloantisera against Antigens Coded for by Defined Regions of the H-2 Complex on PFC Responses In Vitro. The effects of the alloantisera against antigens coded for by defined regions of the H-2 complex on 5-day PFC responses by spleen cells from several strains of mice were determined (Table II). These sera were added to cultures at initiation at a final dilution of 1/50 which did not affect viable cell recovery. Two sera against serological specificity 33 of the H-2K region, which theoretically also could have activity against I region antigens,
effectively suppressed IgM and IgG PFC responses by C57BL/6 spleen cells but did not suppress responses by SJL or B10.S spleen cells. These sera suppressed responses by (C57BL/6 × B10.S)F1 spleen cells approximately 50%. One serum against serological specificity 19 of the $H^{-2^a} K$ region, which theoretically could also have activity against $S$ region antigens, suppressed IgM and IgG PFC responses by SJL, B10.S, and (C57BL/6 × B10.S)F1 spleen cells but failed to suppress responses by C57BL/6 spleen cells. The A.TL anti-A.TH serum, theoretically reactive against $H^{-2^a} I$ region alloantigens, selectively suppressed only IgG PFC responses by SJL, B10.S, and (C57BL/6 × B10.S)F1 spleen cells about 50% and had no effect on PFC responses by C57BL/6 spleen cells. Adding this serum to cultures in higher concentrations resulted in significant toxicity, thus precluding further analysis of the phenomenon. The remaining alloantisera, reactive against serological specificities of the $H^{-2^a} D$ region, failed to suppress responses in any of the spleen cell cultures. In other experiments (data not

| Mouse strain | DBA/2 anti-C57BL/6 | C3H anti-SJL | SJL anti-C57BL/6 | C57BL/6 anti-SJL |
|-------------|-------------------|--------------|-----------------|-----------------|
| C57BL/6     | M 24              | 93           | 30              | 129             |
|             | G 12              | 89           | 14              | 108             |
| SJL         | M 119             | 33           | 107             | 30              |
|             | G 89              | 13           | 87              | 18              |
| A/J         | M 90              | 117          | 122             | 107             |
|             | G 100             | 103          | 96              | 97              |
| A.Sw        | M 111             | 27           | 109             | 24              |
|             | G 138             | 23           | 108             | 23              |
| B10.S       | M 24              | 29           | 19              | 30              |
|             | G 10              | 14           | 16              | 19              |
| (C57BL/6 × SJL)F1 | M 19             | 21           | 25              | 30              |
|             | G 24              | 15           | 19              | 24              |
| (C57BL/6 × B10.S)F1 | M 20            | 30           | 19              | 30              |
|             | G 20              | 20           | 16              | 19              |
| (B10.A × B10.S)F1 | M 24            | 40           | 22              | 31              |
|             | G 17              | 24           | 19              | 20              |

* Final dilution of alloantiserum in cultures was 1/100.
‡ M, IgM; G, IgG PFC response.
TABLE II

Effect of Alloantisera Against Alloantigens Coded for by Defined Regions of the H-2 Complex on PFC Responses In Vitro by Spleen Cells from Various Mouse Strains

| Alloantiserum* | C57BL/6 | SJL | B10.S | (C57BL/6 × B10.S)F1 |
|---------------|---------|------|-------|----------------------|
|               | M†      | G    | M     | G                    | M   | G    | M   | G |
| Anti-33, ? I§  | 16      | 13   | 88    | 89                   | 134 | 173  | 44  | 45 |
| Anti-33, ? I‖  | 37      | 38   | 97    | 109                  | 122 | 102  | 53  | 50 |
| Anti-19, ? S†  | 103     | 111  | 40    | 28                   | 37  | 29   | 38  | 37 |
| Anti-1**       | 103     | 96   | 117   | 51                   | 85  | 44   | 87  | 56 |
| Anti-13, 28, 29, 30‡‡ | 131  | 146  | 127   | 108                  | 117 | 121  | —   | — |
| Anti-2‡§       | 102     | 116  | 104   | 86                   | 110 | 99   | 127 | 109|
| Anti-2, ?S, I‖ | 144     | 119  | 70    | 83                   | 126 | 137  | 131 | 113|
| Anti-2, ? non-H-2‡‡ | 121  | 125  | 131   | 111                  | 131 | 107  | —   | — |
| Anti-19, ?S + anti-33, ?I§ | —     | —    | —     | —                   | —   | —    | —   | — |

The H-2 complex***

| Haplotype  | K region | I region | S region | D region |
|------------|----------|----------|----------|----------|
| H-2o       | 33 (35, 36)‡‡‡ | b     | h/o     | 2(5, 6, 27, 28, 29) |
| H-2o       | 19 (5)   | s       | h/a     | 12(1, 3, 6, 7, 36, 42) |

* Final dilution of alloantiserum in cultures was 1/50.
† M, IgM; G, IgG PFC response.
§ (B10.D2 × A)F1, anti-B10.A (5R), D. Shreffler.
‖ (B10.D2 × A)F1, anti-B10.A (5R), G. Snell.
† A anti-A.TL, D. Shreffler.
** A.TL anti-A.TH (early bleeding, absorbed with A/J thymocytes), D. Shreffler.
‡‡ (AKR × A.CA)F1, anti-AKR.M, G. Snell.
‡§ (B10.D2 × A)F1, anti-HTH, D. Shreffler.
¶ (B10.D2 × A)F1, anti-B10.A(4R), D. Shreffler.
¶¶ (Balb/C × [C × BD]F1, anti-HTG, G. Snell.
*** Modified from: J. Klein and D. C. Shreffler. 1971. The H-2 model for the major histocompatibility systems. Transplant. Rev. 6:3. J. Klein, P. Demant, F. Festenstein, H. O. McDevitt, D. C. Shreffler, G. D. Snell, and J. H. Stimpfling. 1974. Genetic nomenclature for the H-2 complex of the mouse. Immunogenetics. In press.
‡‡‡ The public serological H-2 specificities for K and D regions are in parentheses.

shown), another high titer alloantiserum against H-2o D region serological specificities failed to suppress PFC responses by A/J spleen cells. When the "anti-19" and "anti-33" sera were both added to cultures of (C57BL/6 × B10.S)F1 spleen cells at dilutions of 1/50 the suppression of PFC responses was greater than when either serum was added alone at a dilution of 1/50 or 1/25.

Cellular Site of Action of Alloantisera in Suppression of PFC Responses to SRBC In Vitro. To analyze the cellular sites of action of the suppressive alloantisera, Mφ and lymphoid cells were separated from suspensions of spleen
cells from C57BL/6 (H-2\(^b\)), SJL (H-2\(^s\)), and (C57BL/6 × SJL)F\(_1\) (H-2\(^{b,s}\)) mice and recombined in all the possible combinations. The cultures were stimulated with 10\(^7\) SRBC and DBA/2 anti-C57BL/6 (anti-b) or C3H anti-SJL (anti-s) serum (final dilution, 1/100) was added to one-third the cultures of each combination at initiation. Day 5 IgM and IgG PFC responses are expressed as percent control response (Fig. 4). First, IgM and IgG PFC responses were suppressed by the alloantisera directed against alloantigens on both the M\(^\phi\) and lymphoid cells but not by the irrelevant alloantisera (groups I and II). Second, when the M\(^\phi\) and lymphoid cells were from H-2\(^{b,s}\) F\(_1\) hybrid mice, the anti-b and anti-s alloantisera both suppressed IgM and IgG PFC responses (group III). In all the remaining combinations of M\(^\phi\) and lymphoid cells, when the alloantisera was directed against alloantigens only on M\(^\phi\), both IgM and IgG responses were suppressed. However, when alloantigens only on lymphoid cells were the target of the alloantisera, IgG responses were selectively suppressed. In some combinations of M\(^\phi\) and lymphoid cells, the target cell of

| Group | M\(^\phi\) | Lymphoid | Alloantisera |
|-------|-----------|----------|--------------|
| I     | b         | b        | Anti-b, Anti-s |
| II    | s         | s        | Anti-b, Anti-s |
| III   | b/s       | b/s      | Anti-b, Anti-s |
| IV    | b         | s        | Anti-b, Anti-s |
| V     | s         | b        | Anti-b, Anti-s |
| VI    | b/s       | b        | Anti-b, Anti-s |
| VII   | b/s       | s        | Anti-b, Anti-s |
| VIII  | b         | b/s      | Anti-b, Anti-s |
| IX    | s         | b/s      | Anti-b, Anti-s |

Fig. 4. Suppression of PFC responses by alloantisera against leukocyte alloantigens: site of action. M\(^\phi\) and lymphoid cells from C57BL/6 (b), SJL (s) and (C57BL/6 × SJL)F\(_1\) (b/s) mice were incubated in indicated combinations; DBA/2 anti-C57BL/6 (anti-b) or C3H anti-SJL (anti-s) serum was added to one-third of the cultures at initiation at a final dilution of 1/100. Day 5 PFC responses are expressed as percent control responses in untreated cultures.
the alloantiserum could not be determined with certainty, e.g. groups VI and VIII, cultures with the anti-b serum, or groups VII and IX, cultures with the anti-s serum. Although not evident from this figure, each lymphoid cell population developed comparable PFC responses in control cultures when incubated with syngeneic or allogeneic Mφ. Also, none of the separated Mφ or lymphoid cell populations alone developed significant PFC responses (data not shown). Thus, alloantisera can apparently suppress PFC responses by interfering with the functions of macrophages or by interfering with the interactions among T and B cells which are so crucial for development of IgG responses.

In a modification of this approach, separated C57BL/6 Mφ and lymphoid cells were incubated with or without DBA/2 anti-C57BL/6 serum at a final dilution of 1/25 for 24 h. Then, the lymphoid cells were harvested, washed, adjusted to 10 × 10⁶ cells/ml, and added with 10⁷ SRBC to appropriate dishes containing adherent Mφ which had also been washed with HBSS. Cultures of normal or alloantiserum-treated Mφ or lymphoid cells incubated alone with SRBC failed to develop significant 5-day IgM or IgG PFC responses (Table III). Responses by alloantiserum-treated Mφ and lymphoid cells were effectively suppressed when compared to responses by normal Mφ and lymphoid cells. When alloantiserum-treated Mφ were incubated with normal lymphoid cells, IgM and IgG PFC responses were suppressed to the same degree as responses in cultures where both Mφ and lymphoid cells had been treated with the alloantiserum. In contrast,

### Table III

*Suppression of Plaque-Forming Cell Responses to SRBC In Vitro by Alloantiserum: Site of Action*

| Culture variables: | PFC/culture* |
|--------------------|--------------|
| 10⁶ C57BL/6 Mφ     |              |
| 10 × 10⁶ C57BL/6   |              |
| lymphoid cells     |              |

| Normal | 1,850 (100)$ | 555 (100) |
|--------|--------------|-----------|
| Normal | 18 (1<1)     | 5 (<1)    |
| Treated| 185 (12)     | 38 (7)    |
| Normal | 142 (7)      | 30 (5)    |
| Treated| 655 (35)     | 215 (42)  |
| Treated| 770 (41)     | 235 (41)  |
| Normal | 1,600 (86)   | 525 (92)  |

*PFC/culture on day 5 from a representative experiment.
‡Separated C57BL/6 Mφ and lymphoid cells were incubated without (normal) or with (treated) DBA/2 anti-C57BL/6 serum at a final dilution of 1/25 for 24 h. Mφ adherent to culture dishes were washed with HBSS; lymphoid cells were harvested, washed, adjusted to 10 × 10⁶/ml, and added to appropriate dishes containing Mφ and 10⁷ SRBC. The numbers of Mφ in normal and treated culture dishes were comparable.
§The numbers in parentheses are the mean percent of control responses from five experiments.
cultures of normal Mφ and alloantiserum-treated lymphoid cells developed responses which were not significantly suppressed. Viable cell recovery from all cultures was approximately $2 \times 10^6$ cells. Thus, a 24-h exposure to alloantiserum renders Mφ incapable of supporting development of PFC responses, whereas, lymphoid cells similarly exposed are able to develop normal responses.

Effects of Alloantiserum on Antigen Binding by Mφ and on the Ability of Mφ-Bound Antigen to Stimulate PFC Responses In Vitro. To further probe the sites and mechanisms of suppression of PFC responses by alloantiserum, we have used GAT as our antigen. Antibody responses to GAT are controlled by an H-2-linked immune response (Ir) gene which maps within the H-2 complex (8, 14). Spleen cells from C57BL/6 mice, a responder strain, but not from SJL mice, a nonresponder strain, develop IgG PFC responses to GAT in vitro (11). Furthermore, Mφ from either C57BL/6 or SJL mice which have been reacted with GAT and bear nanogram quantities of GAT stimulate comparable GAT-specific PFC responses by responder C57BL/6 lymphoid cells (4). Other studies have demonstrated that the defect in genetic nonresponder spleen cells is the inability of their T cells to provide appropriate helper function for B cells which, however, can develop PFC responses to GAT under appropriate circumstances (4, 8, 11, 15).

Several features of alloantiserum-mediated suppression of GAT-specific PFC responses by C57BL/6 lymphoid cells are shown in Table IV. First, $7 \times 10^4$ C57BL/6 Mφ bearing 3.17 ng of GAT (Mφ pulsed with GAT as described in the Materials and Methods) stimulated GAT-specific IgG PFC responses by C57BL/6 lymphoid cells comparable in magnitude to responses stimulated by 10 μg of soluble GAT (4, 12, see also Table V). This response was suppressed by antiserum against C57BL/6 alloantigens but not by antiserum against SJL alloantigens when the antiserum was added to cultures at initiation at a final dilution of 1/100. Second, when C57BL/6 Mφ were reacted with either DBA/2 anti-C57BL/6 or C3H anti-SJL alloantiserum before and during the reaction with GAT, the amount of GAT bound/7 × 10^4 Mφ was not reduced. However, GAT-Mφ which had been reacted with the anti-C57BL/6 alloantiserum failed to stimulate a PFC response by the C57BL/6 lymphoid cells, whereas GAT-Mφ which had been reacted with the anti-SJL alloantiserum stimulated normal responses. Third, when alloantiserum-treated Mφ bearing no GAT were added to cultures of C57BL/6 lymphoid cells and GAT-Mφ which had not been reacted with alloantiserum, normal GAT-specific PFC responses developed. Viable cell recovery from all cultures was approximately $2 \times 10^6$ cells. These experiments allow several conclusions: (a) alloantiserum does not interfere with antigen uptake by Mφ but does interfere with the ability of Mφ-bound antigen to stimulate PFC responses; (b) alloantiserum does not interfere with the viability promoting function of Mφ (12); and (c) alloantiserum-mediated suppression is not due to soluble antigen-antibody complexes from Mφ interfering with the responses of the lymphoid cells.

The experiments shown in Table V further extend the analysis of the site of action of suppressive alloantiserum in the GAT system. As shown in the top half of the table, C57BL/6 Mφ bearing 1.46 ng GAT and SJL Mφ bearing 0.92 ng GAT
TABLE IV
Effect of Alloantiserum on Antigen Binding by Macrophages and on the Ability of Macrophage-Bound Antigen to Stimulate Plaque-Forming Cell Responses In Vitro

| Culture variables: | 10 × 10⁴ C57BL/6 Lymphoid Cells* | 7 × 10⁴ alloantiserum-treated GAT-Mφ§ | 7 × 10⁴ alloantiserum-treated Mφ* | Alloantiserum | GAT-specific IgG PFC/culture‡ |
|-------------------|-------------------------------|-----------------------------------|---------------------------------|--------------|-------------------------------|
|                   |                               | GAT-Mφ||                     |                  | None                           | 880 (100)** |
| +                 | −                             | −                                 | N/A                            |              | DBA anti-C57]] added to culture| 40 (6)      |
| +                 | −                             | −                                 | C3H anti-SJL|| added to culture | 740 (87)    |
| −                 | + (6.89 ng)                   | −                                 | DBA anti-C57                 | 0            (5) |
| +                 | −                             | +                                 | C3H anti-SJL                  | 730 (92)     |
| +                 | −                             | +                                 | DBA anti-C57                 | 830 (97)     |
|                   |                               | +                                 | C3H anti-SJL                  | 790 (90)     |

* Lymphoid cells incubated with 10 µg soluble GAT failed to develop significant PFC responses. ‡ GAT-specific PFC/culture on day 5 from a representative experiment.
§ C57BL/6 peritoneal exudate Mφ were pulsed with GAT as described in the Materials and Methods; in this experiment 7 × 10⁴ Mφ bound 3.17 ng GAT.
|| Mφ were reacted with the indicated alloantiserum at a final dilution of 1/100 at 4°C for 30 min before reaction with GAT. Thereafter, Mφ were treated as described in the Materials and Methods; numbers in parentheses are the ng of GAT/7 × 10⁴ Mφ.
* Mφ were treated with the indicated alloantiserum as described above but were not pulsed with GAT.
** The numbers in parentheses are the mean percent of control responses from three experiments.
]] Alloantiserum added to these cultures at initiation at a final dilution of 1/100.

stimulated comparable PFC responses by C57BL/6 lymphoid cells. Anti-C57BL/6 but not anti-SJL alloantiserum suppressed the response stimulated by C57BL/6 GAT-Mφ. However, when SJL GAT-Mφ were used, both alloantisera suppressed the PFC response. In the case of the anti-C57BL/6 alloantiserum, the site of action can only be the lymphoid cells, whereas with the anti-SJL alloantiserum, the only site of action can be at the GAT-Mφ. The experiment shown in the bottom half of the table illustrates that Mφ after brief exposure to alloantiserum which reacts with membrane alloantigens did not recover the ability to present antigen in a manner which stimulated PFC responses by the lymphoid cells.

Discussion

These experiments document several of the salient features about the phenomenology and mechanisms involved in the suppression of PFC responses to SRBC and GAT by mouse spleen in vitro by alloantisera against leukocyte alloantigens.
These experiments demonstrate that alloantisera against antigens encoded by K and I region genes of the H-2 complex, and antisera to non-H-2 antigens, either minor histocompatibility antigens or other leukocyte alloantigens can effectively suppress PFC responses in vitro. Furthermore, these alloantisera suppressed both IgM and IgG PFC responses when directed against alloantigens on Mφ, but suppressed only IgG responses when directed against antigens on lymphoid cells. These experiments further emphasize the importance of membrane alloantigens in mediating the interactions among antigen, Mφ, T cells, and B cells which are so crucial for the initiation of PFC responses to complex, multideterminant antigens in vitro. The mode of action of these suppressive alloantisera, from a

| Table V |
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Suppression of Plaque-Forming Cell Responses to GAT In Vitro by Alloantisera: Site of Action

| Culture variables: | 10 × 10⁴ C57BL/6 lymphoid cells* | GAT-specific IgG PFC/culture‡ |
|---|---|---|
| 7 × 10⁴ GAT-Mφ§ | Autoantiserum added to culture| |
| C57 | None | 305 (100)* |
| C57 | DBA anti-C57 | 35 (13) |
| C57 | C3H anti-SJL | 340 (115) |
| SJL | None | 380 (100) |
| SJL | DBA anti-C57 | 80 (25) |
| SJL | C3H anti-SJL | 40 (12) |

| 7 × 10⁴ Mφ | Mφ pretreatment** | 10 μg of GAT |
|---|---|---|
| C57 | None | 565 (100) |
| C57 | DBA anti-C57 | 30 (7) |
| C57 | C3H anti-SJL | 455 (85) |
| SJL | None | 425 (100) |
| SJL | DBA anti-C57 | 605 (133) |
| SJL | C3H anti-SJL | 0 (6) |

* Separated lymphoid cells incubated with 10 μg GAT failed to develop significant PFC responses.
‡ GAT-specific PFC/culture on day 5 from a representative experiment.
§ C57BL/6 or SJL peritoneal exudate Mφ were pulsed with GAT; in this experiment there were 1.46 ng GAT/7 × 10⁴ C57BL/6 Mφ and 0.92 ng GAT/7 × 10⁴ SJL Mφ.
‖ Alloantiserum was added to cultures at initiation at a final dilution of 1/100.
¶ The numbers in parentheses are the mean percent of control responses from three experiments.
** Mφ were reacted with the indicated alloantiserum at a final dilution of 1/100 at 4°C for 30 min, washed, and 7 × 10⁴ Mφ were added to cultures in 0.1 ml HBSS.
phenomenological point of view, is not unlike that of anti-\(\mu\)-chain antisera (16), and antibody to the stimulating antigen (10), both of which mediate their effect early in a specific, noncytotoxic fashion and interfere with the successful initiation of the immune response. The possibility that alloantisera suppress PFC responses in a nonspecific fashion by combining with any membrane alloantigens on responding spleen cells and producing membrane perturbations which preclude development of immune responses can be ruled out since antisera against \(H-2D\) region alloantigens failed to suppress responses.

In view of the demonstrated critical importance of molecules encoded by the K and I regions of the \(H-2\) complex in determining successful physiologic cooperation between T cells and B cells in antibody responses (7, 8), we were somewhat surprised that antibodies against non-\(H-2\) alloantigens could also effectively suppress PFC responses in vitro. Furthermore, the effects of the various alloantisera on PFC responses by \(F_1\) hybrid spleen cells were also unexpected in the sense that they suggest that the \(H-2\) and non-\(H-2\) alloantigens on \(F_1\) hybrid cells which have not reacted with alloantiserum are unable to mediate the cell interactions required for development of PFC responses. The fact that the polyspecific alloantisera suppressed responses by \(F_1\) hybrid and parental spleen cells to the same degree, whereas, antisera to K and I region alloantigens suppressed responses by \(F_1\) hybrid spleen cells less efficiently than responses by parental spleen cells at the same serum dilution may be due to the different titers and immunoglobulin classes of the various antisera or to the fact that the polyspecific antisera reacted with non-\(H-2\) as well as \(H-2\) antigens. If the latter is true, it suggests that non-\(H-2\) alloantigens may play a more significant role in mediating the essential cell interactions in the development of antibody responses than has previously been realized. Studies to determine the identity and function(s) of such non-\(H-2\) alloantigens in the development of antibody responses are in progress.

The polyspecific antisera against \(H-2\) and non-\(H-2\) alloantigens and the antisera specific for K region alloantigens suppressed both IgM and IgG PFC responses suggesting that these antisera are interfering with cell interactions which are fundamental to the development of all antibody responses. In contrast, the antiserum against alloantigens coded for by only the I region of \(H-2^s\) (A.TL anti-A.TH) selectively suppressed IgG PFC responses by \(H-2^s\) spleen cells. Since this antiserum had no effect on IgM PFC responses, it seems likely that its mechanism(s) of action is quite different from that of the other alloantisera.

The observations that the polyspecific alloantisera interfere in a rather long-lasting fashion with the ability of Mφ to stimulate development of both IgM and IgG PFC response, but not at all with their ability to bind antigen or their viability promoting function (12), may account for the ability of these antisera to prevent successful initiation of the antibody responses. Why are the alloantiserum-treated Mφ with associated antigen unable to stimulate lymphoid cells to develop an antibody response? One possibility is that the membrane of the antigen-bearing Mφ must have a "freedom of movement" to effectively stimulate

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2 Detailed investigations on the suppressive effects of A.TL anti-A.TH sera obtained from latter bleedings in the immunization schedule are in progress and will be reported separately.
lymphoid cells and that the alloantiserum restricts this freedom. It has also been suggested that the relevant Mφ-associated antigen must first be endocytosed, partially degraded in phagolysosomes, and returned to the membrane before it is effectively immunogenic (17). Thus, the alloantiserum could interfere with this phase of antigen handling by Mφ.

In addition to interfering with Mφ handling of antigen, alloantiserum could also block the appropriate required interactions of Mφ with T and B cells. In this sense, the suppression of PFC responses we observe may be analogous to the alloantiserum-mediated inhibition of antigen-specific DNA synthetic responses by guinea pig lymphocytes (18). In that experimental system, alloantiserum can suppress by interfering with the function of the receptor for antigen on T cells, or by interfering with interactions between antigen-bearing Mφ and T cells which are controlled by gene products of the major histocompatibility complex including \( Ir \) genes (19, 20). In the guinea pig, Mφ and T cells must be syngeneic for successful development of DNA synthetic responses to antigen (19), whereas, in the mouse, it is the T and B cells, and not the Mφ, which must be syngeneic or semisyngeneic for successful development of antibody responses (8). These differences in the requirements for syngenicity of Mφ and the responding lymphoid cells may depend on which type of lymphoid cell response is being measured and its sensitivity to activation. For DNA synthetic responses by T cells, it may be that Mφ and T cells must be syngeneic for optimal activation of T cells to proceed. Similarly, for antibody responses by B cells, it may be that T and B cells must be syngeneic for effective triggering of the B cells to occur. However, allogeneic Mφ may be able to activate T cells sufficiently for effective cooperation with syngeneic B cells, but not for development of DNA synthetic responses.

Whether it is the T cell, B cell, or the interactions among these two cells which is the site of action of the polyspecific alloantisera on the lymphoid cells cannot be discerned from the present data. However, the selective suppression of IgG responses by antisera against alloantigens on lymphoid cells indicates that interactions involved in the generation of IgG responses are affected more than interactions involved with IgM responses. Two mechanisms can be dismissed: (a) the alloantiserum is not interfering with antigen recognition by B cells, since this function is mediated by immunoglobulin receptors (16, 21), and normal IgM responses do develop; and (b) the alloantiserum is probably not activating a population of nonspecific suppressor T cells, since such cells do not interfere with initiation of the PFC response (22) which the alloantiserum does. Furthermore, we have been unable to detect suppressor T-cell activity after interaction of spleen cells with these alloantisera.\(^3\)

It is conceivable that the alloantisera interfere with antigen recognition by T cells. The precise nature of the T-cell receptor for antigen is still unknown; immunoglobulin, possibly a monomeric IgM molecule (21), and products of \( Ir \) genes (8) have been proposed. If \( Ir \)-gene products are the receptors, these polyspecific alloantisera, which should be reactive with alloantigens coded for by the I region of the \( H-2 \) complex, could block antigen recognition directly by...\(^3\)

\(^3\) Pierce, C. W. Unpublished observations.
interfering with the function of the receptor. However, this would be obtained only if the Ir-gene product receptor and the target alloantigens were identical or were spatially related on the cell membrane. If immunoglobulin is the receptor and the alloantigens against which antisera are reactive are closely juxtaposed on the T-cell membrane, the alloantisera could interfere with antigen recognition in a steric fashion.

In view of the requirement that T and B cells must be syngeneic or at least semisyngeneic for successful development of antibody responses (usually of the IgG class) by B cells, it is highly likely that the alloantisera act by interfering with the necessary interactions between the T cells and B cells. The membrane molecules involved in these interactions could be H-2 antigens or alloantigens coded for by the H-2 I region (Ia antigens) (8), both of which have been demonstrated on membranes of T and B cells (14, 23-26). If the antisera react with antigens on T cells, these cells may be unable to efficiently interact with B cells. Alternatively, if the alloantisera interacts with these antigens on B cells, which have been proposed as the "acceptor site" for the second signal from T cells (8), the T cells or their products so essential for stimulation of IgG responses would be denied access to the B cells and suppression of IgG responses would be the result. The antisera could also react with antigens on both T and B cells to preclude their effective interaction.

In conclusion, we have demonstrated that alloantisera against non-H-2 alloantigens and alloantigens coded for by the K region of the H-2 complex suppressed both IgM and IgG PFC responses in vitro, whereas, antisera against alloantigens coded for by the I region selectively suppressed IgG PFC responses. The polyclonal alloantisera used in this study may contain antibodies to alloantigens coded for by non-H-2, H-2K, and H-2I region genes. Indeed, Sachs and Cone (24) and David and Shreffler (27) have identified antibodies to I region antigens in conventional anti-H-2 alloantisera. In view of the dual sites of action at the cellular level for suppressive activity of the polyclonal alloantisera which have been demonstrated in this study, it is reasonable to speculate that the antibodies against non-H-2 alloantigens and alloantigens coded for by the K region of the H-2 complex which suppress both IgM and IgG responses act by interfering with MΦ function, while selective suppression of the IgG responses is due to antibody to I region alloantigens which affect lymphocyte function. Investigations of these possibilities are currently in progress.

Summary

The effects of alloantisera against leukocyte alloantigens on plaque-forming cell (PFC) responses to sheep erythrocytes and the terpolymer of L-glutamic acid6°-L-alanine3°-L-tyrosine1° (GAT) by mouse spleen cells in vitro have been investigated. Polyclonal antibodies against both H-2 and non-H-2 alloantigens on responding spleen cells suppressed both IgM and IgG PFC responses; antisera against alloantigens coded for by the K and I regions, but not the D region, of the H-2 complex also effectively suppressed PFC responses. The suppression was not due to cytotoxicity to the spleen cells or anti-immunoglobulin activity in the
sera and was directly related to the amount of antiserum added to the cultures. The suppression was specific for spleen cells against which the alloantisera was directed. The alloantisera suppressed responses most effectively when present during the first 24 h of incubation, and although not rendering lymphoid cells incapable of developing PFC responses after removal of noncell-bound antibody, did act by interfering with successful initiation of the PFC response. The alloantisera suppressed both IgM and IgG PFC responses when directed against alloantigens only on macrophages, but selectively suppressed IgG responses when directed against alloantigens only on lymphoid cells. The alloantisera did not interfere with the ability of macrophages to bind GAT or to support the viability of the lymphoid cells, but did interfere with the ability of macrophage-associated antigen to effectively stimulate antibody responses by the lymphoid cells. Possible mechanisms for the effects of alloantisera on macrophages and the selective suppression of IgG responses when the antisera are directed against alloantigens on lymphoid cells are discussed with reference to our current understanding of genetic restrictions governing cell interactions in the development of antibody responses in mice.

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