Induction of Cytotoxic Factors by Immunization of Mice with Freund’s Adjuvant Components

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ABSTRACT

Antidiotypic antibody (AIA) was raised in mice by immunization with MOPC 315 immunoglobulin A emulsified in Freund’s adjuvant (FA). The antibody content of mouse serum was assessed by (a) its ability to inhibit rosetting of 2,4,6-trinitrophenyl-sheep red blood cells around MOPC 315 myeloma cells, and (b) by a solid phase antigen-binding plate assay based on reactivity with 125I-Protein A and inhibition in the presence of dinitrophenyl aminocaproic acid. FA was necessary for the production of AIA to MOPC 315 immunoglobulin A. Some of the AIA-containing mouse sera were cytotoxic for MOPC 315 cells in the presence of guinea pig complement. However, cytotoxicity was not correlated with the amount of AIA, as assessed by inhibition of rosette formation, nor was it specific for myeloma cells bearing the MOPC 315 idiotype. Furthermore, cytotoxicity could also be generated by immunization of mice with complete Freund’s adjuvant, incomplete Freund’s adjuvant, or the muramyl dipeptide portion of mycobacteria, all in the absence of MOPC 315 immunoglobulin A. Therefore, the complement-dependent cytotoxic antibodies in the AIA-containing antisera, which belonged to the immunoglobulin G and M classes, were likely directed against some component of FA. Myeloma cells which were not killed by anti-FA antisera, as assessed by dye exclusion, were inhibited in their ability to secrete immunoglobulin and to form clones in agar.

INTRODUCTION

We have been interested in killing mouse myeloma cells with AIA and complement, to select for mutants in immunoglobulin production. Similar approaches based on humoral cytotoxicity have been used by a number of workers to demonstrate mutants of myeloma cells and other transplantable tumors (4, 5, 10, 17, 21). We have found in the present studies that our goal was not achieved by the presence of cytotoxic factors for mouse myeloma cells which are produced as a result of immunization with CFA, IFA, or the MDP portion of mycobacteria. Since FA must be administered together with immunoglobulin for the production of AIA, the question is raised whether AIA’s possess any cytotoxicity for mouse myeloma cells. A number of recent studies have suggested that AIA’s are not cytotoxic in vitro, as assessed by dye exclusion (7, 11, 12, 25). The present experiments provide further evidence that the cytotoxicity observed in AIA-containing sera results from complement-dependent cytotoxic factors, which are presumably antibodies directed against some component of FA.

MATERIALS AND METHODS

Cultured Myeloma Cell Lines. Mouse myeloma cells were maintained as suspension cultures in Petri dishes containing α-growth medium (Flow Laboratories, Inc., McLean, Va.) supplemented with 15% fetal calf serum, 2 mM glutamine, and a penicillin-streptomycin mixture. The cells were grown at 37°C in a humidified CO2 incubator and were used either to form rosettes (MOPC 315) or as targets in cytotoxicity tests (all myeloma lines). The cell lines included MOPC 315 (IgA), MOPC 315 clone 36 (non-immunoglobulin-producing variant), MOPC 31C (IgG1), and P3 (IgG2a).

Purification of Mouse Myeloma Immunoglobulin. Ascitic fluid was obtained by tapping the peritoneal cavities of BALB/c mice bearing tumors that formed following injection of MOPC 315 myeloma cells. Purified MOPC 315 IgA was obtained as described previously (13).

Immunization of Mice. Eight-week-old female BALB/c mice were purchased from Canadian Breeding Laboratories (Montreal, Quebec, Canada). Preimmune serum was obtained by bleeding from the retroorbital sinus. The mice were then immunized at weekly intervals s.c. and into the foot pads with 200 µg of MOPC 315 IgA in 0.6 ml, according to the schedule of Sakato and Eisen (23) (Table 1, Schedule 1). In some experiments, IFA was used instead of CFA (Schedule 2), and, in others, immunoglobulin was administered in PBS (Schedule 3). In a further series of experiments, no immunoglobulin was injected, and only adjuvant was used for immunization (Schedules 4 and 5). An additional group of mice was immunized with the MDP portion of mycobacteria, kindly provided by Dr. L. Chedid of the Pasteur Institute (Paris, France) (6). MDP was dissolved in either PBS or IFA and administered s.c. at a dose of 100 µg/injection (Schedules 6 and 7). The mice were bledd from the retroorbital sinus 1 week following the last injection. The antisera were heat inactivated at 56°C for 30 min and frozen in aliquots at −70°C until used. In some experiments, antisera obtained from mice immunized with FA was absorbed with an equal volume of packed Mycobacterium tuberculosis organism (Difco Laboratories Inc., Detroit, Mich.) for 1 hr at room temperature or precipitated at equivalence with rabbit anti-mouse IgG or anti-IgM (Litton Bionetics, Kensington, Md.) prior to use in cytotoxicity experiments.

Cytotoxicity. A microcytotoxicity technique was used to test the effect of various mouse antisera on mouse myeloma cells.
Viable myeloma cells (>95%) were isolated on a Isopaque-Ficol gradient and diluted to 5 × 10^5 cells/ml with PBS. Two µl (1000 cells) were dispensed into wells of a Terasaki tissue culture plate (Falcon Model 3034; Falcon Plastics, Oxnard, Calif.), followed by 2 µl of various dilutions of the mouse antiserum to be tested for cytotoxicity. After a 20-min incubation period at 4°C, the antiserum was removed, and 2 µl of undiluted guinea pig complement (Grand Island Biological Co., Grand Island, N. Y.) were added to the wells. Following a further incubation at 37°C for 45 min, the complement was removed, and the wells were flooded with 0.2% nigrosin black. After standing at 4°C for 30 min, the dye was removed, and counts of cell viability were made. Cytotoxic index was expressed as the percentage of dead cells. Controls consisting of myeloma cells alone, cells treated with antiserum alone, or complement alone showed a viability greater than 95%.

**Antigen-binding Plate Assay for Antibody to MOPC 315 IgA.** An antigen-binding plate assay was used for the detection of mouse anti-MOPC 315 IgA antibodies (8). Wells of disposable microtiter plates (Cooke 1-220-24; Dynatech Laboratory, Alexandria, Va.) were coated with 50 µl of MOPC 315 IgA (10 µg/ml) in PBS for 2 hr at room temperature. The immunoglobulin was then removed, and the wells were flooded with a 10% solution of fetal calf serum for a further 2 hr at 37°C, following which the fetal calf serum was removed and 50 µl of various dilutions of mouse anti-MOPC 315 IgA antiserum were added to the wells. The plates were incubated for 16 hr at 4°C, at which time the antiserum was removed, and the wells were washed with PBS. Fifty µl of 125I-Protein A, containing approximately 10,000 cpm, were added to the wells for a further 2 hr at room temperature. The wells were washed with PBS, cut into individual samples, and counted with a Beckman 300 gamma counter. In some experiments, dilutions of antiserum and 0.1 M DNP-aminocaproic acid were added simultaneously.

**Assays of Plaque and Clone Formation by Myeloma Cells.** Myeloma cells were plated in agar using modifications of a method previously described (14). A base layer of 5 ml agar-growth medium was allowed to solidify in 60-mm diameter tissue culture dishes. This was overlaid with 400 myeloma cells in 1 ml of an agar-growth medium mixture containing 1.5% sheep RBC conjugated to Protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) (14). After incubation for 3 days at 37°C in a humidified CO2 incubator, 1 ml of a 1:512 dilution of rabbit anti-mouse immunoglobulin antiserum was added to the dishes, and on the next day 0.2 ml of a 1:4 dilution of guinea pig complement were added. Over the next 2 to 4 hr, macroscopically visible clear areas (plaques) formed around viable myeloma cell colonies which were secreting immunoglobulin, and these were counted. The plates were then returned to the incubator for a further 10 days, at which time the colonies had grown into macroscopically visible clones, and these were counted.

To test the effect of mouse anti-FA or anti-MDP antiserum and complement on the ability of myeloma cells to form plaques and clones, 5 × 10^5 myeloma cells in 0.1 ml were incubated at 4°C with 0.1 ml of mouse antiserum. The cells were then spun and incubated at 37°C with 0.1 ml of a 1:2 dilution for guinea pig complement prior to plating. Controls consisted of myeloma cells treated with antiserum minus complement.

**Rosette Formation and Inhibition Using Mouse Anti-MOPC 315 IgA Antiserum.** TNP was conjugated with sheep RBC according to the method described by Hannestadt et al. (15). Rosette formation was tested by mixing 1 × 10^5 myeloma cells in 0.1 ml with 0.04 ml of a 0.5% solution of TNP-sheep RBC. The mixture was centrifuged at 700 rpm for 10 min, incubated at room temperature for 30 min, and resuspended in 1 drop of 0.2% Trypan blue. Rosettes were defined as 3 or more RBC forming around a myeloma cell, and there were always greater than 90% present in the case of the IgA-producing MOPC 315 cells and less than 2% with the non-IgA clone 36 variant of MOPC 315. To test what effect mouse anti-MOPC 315 IgA antiserum had on rosette formation, MOPC 315 myeloma cells were treated with 10 µl of antiserum for 30 min at 4°C and washed prior to the addition of TNP-sheep RBC.

### RESULTS

**Lack of a Positive Correlation between AIA Content of Mouse Anti-MOPC 315 IgA Antiserum and Cytotoxicity by Dye Exclusion.** A group of 9 mice was given a complete course of injections of MOPC 315 IgA in FA (Table 1, Schedule 1). The antisera obtained were tested for their ability to inhibit the formation of rosettes around MOPC 315 myeloma cells, using TNP-sheep RBC (Chart 1, top). All of the mouse antiserum inhibited rosette formation, from 58 to 98%, indicating the presence of varying amounts of AIA. The same antiserum were tested for their ability to kill MOPC 315 myeloma cells in the presence of guinea pig complement (Chart 1, bottom). Some sera showed either none or very little cytotoxicity (Antiserum 1, 3, 6, 7, and 8), others showed intermediate cytotoxicity (Antiserum 2, 4, and 5), and one showed strong cytotoxicity (Antiserum 9). Therefore, these studies indicated that there was no positive correlation between the AIA content of these antiserum and their cytotoxic potential.

**Demonstration of AIA in Mouse Anti-MOPC 315 IgA Antiserum by a Solid-Phase Antigen-binding Plate Assay.** The antisera which were tested by rosette inhibition (Chart 1) were also assessed for AIA content by an antigen-binding plate assay (Chart 2). Wells of a microtiter plate were coated with MOPC 315 IgA. Serial dilutions of mouse anti-MOPC 315 IgA antiserum were added to the wells, followed by 125I-Protein A. With all of the antiserum, radioactivity could be demonstrated after exhaustive washing of the wells, and values fell toward the base line with progressive dilution of the antiserum. Normal mouse serum showed no specific binding to antigen-coated wells. When the antigen binding assay was carried out in the

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**Table 1**

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<td>BALB/c mice were given injections s.c. and into the footpads of 200 µg MOPC 315 IgA emulsified in CFA or IFA or dissolved in PBS (Schedules 1 to 3). The IgA was omitted in Schedules 4 to 7. MDP was dissolved in PBS and used at a dose of 100 µg for injection.</td>
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**Cytotoxicity Induced by FA**
presence of the DNP radical, less 125I-Protein A was bound than in its absence. Since MOPC 315 IgA possesses anti-DNP radical specificity (22), this result showed that the antibody present in the antisera was AIA. The presence of CFA or IFA as adjuvant was necessary for the production of AIA. There was no detectable AIA response when animals were immunized with MOPC 315 IgA in PBS (data not shown).

Cytotoxicity by Dye Exclusion of Mouse Anti-MOPC 315 IgA Antiserum for Various Myeloma Cell Lines. The strongly cytotoxic Antiserum 9 of Chart 1 (bottom) was tested for its ability to kill a number of different mouse myeloma cell lines in the presence of guinea pig complement (Chart 3). The antiserum showed marked cytotoxicity not only for the IgA-producing MOPC 315 cell line, but also for the non-immunoglobulin-producing clone 36 variant of MOPC 315 and somewhat less cytotoxicity for the IgG-producing MOPC 31C and P3 cell lines. Therefore, killing by mouse anti-MOPC 315 IgA antiserum was not specific for myeloma cells bearing the MOPC 315 idiootype.

Cytotoxicity by Dye Exclusion of Mouse Anti-Freund's Adjuvant and Anti-MDP Antisera For Myeloma Cells. Serum from mice immunized according to Schedules 4 to 7 (Table 1) were tested for their ability to kill various mouse myeloma cell lines in the presence of guinea pig complement (Chart 4). In the case of most antisera to CFA (Schedule 4), cytotoxicity was observed with the IgA-producing MOPC 315 cells and the non-immunoglobulin-producing clone 36 variant of MOPC 315 (Chart 5, top). Cytotoxicity was not observed with IgG-producing MOPC 31C or P3 cells with some antisera, but cytotoxicity was present when other antisera were used (Charts 3 and 5). Using IgA-producing MOPC 315 myeloma cells as targets, many sera from mice immunized according to Schedules 4, 5, and 6 were toxic (Chart 4, bottom). Sera from mice immunized with MDP emulsified in IFA were also toxic (Schedule 7). The cytotoxic factors could be removed by precipitation of the antisera at equivalence with rabbit anti-mouse IgG and anti-IgM antisera (data not shown). Therefore, these studies indicated that mice immunized with either FA or the MDP portion of mycobacteria contained complement-dependent cytotoxic antibodies (IgM and IgG) in their serum and suggested that the cytotoxicity shown by sera of mice immunized with immunoglobulin in FA was a consequence of an immunological response to some component of the FA. There were no significant differences in the number of animals responding with cytotoxic
antibodies after immunization with IFA or MDP (data not shown).

Cytotoxicity by Dye Exclusion of Mouse Anti-CFA Antiserum Absorbed with Mycobacteria. The preceding studies showed that cytotoxic antibodies were generated in mouse serum as a result of immunization with both the active component of mycobacteria (i.e., MDP) and the ingredients of FA (IFA). This was further confirmed by treating myeloma cells with mouse antiserum to CFA (Schedule 4) which has been absorbed with intact M. tuberculosis organisms (Chart 5). Residual cytotoxicity was seen with the absorbed antiserum, indicating the presence of cytotoxic antibodies with at least 2 specificities.

Cytotoxicity, by assays of Plaque and Clone Formation, of Mouse Antisera to CFA, IFA, and MDP for Mouse Myeloma Cells. The IgG₁-producing P3 cell line was not killed by some anti-FA antisera (Table 1, Schedule 4), as assessed by dye exclusion (Chart 4). The effect of such antisera on the ability of

Chart 4. Cytotoxicity by dye exclusion of serum from mice immunized with FA or the MDP component of mycobacteria. Cytotoxic index (percentage of dead cells) was assessed by nigrosin black exclusion. Top, different mouse myeloma cell lines [MOPC 315 (IgA), MOPC 315 clone 36 (nonproducing variant), MOPC 31C (IgG₁), and P3 (IgG₁)] were treated with guinea pig complement and serum from mice immunized with CFA. Bottom, IgG₁-producing MOPC 315 cells were treated with guinea pig complement and serum from mice immunized with CFA, IFA, MDP, or normal mouse serum.

Chart 5. Cytotoxicity by dye exclusion of unabsorbed mouse anti-FA serum and antiserum absorbed with mycobacteria, for MOPC 31C and MOPC 315 myeloma cells. MOPC 31C and MOPC 315 myeloma cells were treated with guinea pig complement and either serum obtained from mice immunized with CFA or such serum absorbed with M. tuberculosis (Mycobact.) organisms. Cytotoxic index (percentage of dead cells) was assessed by nigrosin black exclusion.

Chart 6. Cytotoxicity assessed by plaque and clone formation of serum from mice immunized with FA or the MDP component of mycobacteria. IgG₁-producing P3 myeloma cells were treated with guinea pig complement and serum from mice immunized with CFA, IFA, or MDP. Four hundred cells were plated in agar containing Protein A-conjugated sheep RBC. Plaque formation was assessed after 4 days by adding rabbit anti-IgG antiserum and guinea pig complement, white clones were counted after 14 days. The values shown represent the mean plaques and clones observed on 3 dishes, and the variation between dishes was less than 10%.

P3 cells to secrete immunoglobulin (plaque formation) and to grow in agar as colonies (clone formation) was assessed (Chart 6) because these assays could be more sensitive indicators of cytotoxicity than could dye exclusion (24). P3 cells treated with mouse antiserum to CFA or IFA and complement formed fewer plaques and clones than did control cells treated with antiserum alone. Treatment of P3 cells with complement and antiserum to MDP had a lesser inhibitory effect on plaque formation but no effect on clone formation. Therefore, these studies showed that although P3 myeloma cells were not killed by some anti-FA antisera, as assessed by dye exclusion (Chart 4), their ability to secrete immunoglobulin and to grow in agar was adversely affected.
DISCUSSION

The present studies have shown that complement-dependent cytotoxic antibodies of the IgG and IgM classes can be detected in mouse serum following immunization with either CFA or IFA (Charts 3 and 4). These antibodies kill myeloma cells, as demonstrated by dye exclusion, and also prevent myeloma cells from secreting immunoglobulin or forming clones in agar (Chart 6). Similar cytotoxic antibodies can be generated in serum by immunization with the MDP portion of mycobacteria, although less of an effect of this antiserum on plaque formation and cloning of myeloma cells was noted. Therefore, these findings would indicate that cytotoxic antibodies with 2 different specificities were produced, one against IFA and the other against MDP. This was confirmed by demonstrating that absorption of a cytotoxic serum (anti-CFA antiserum) with intact mycobacteria resulted in the removal of only a portion of the cytotoxicity (Chart 5). It is also possible that immunization with FA components induces an antibody response to host tissue antigens such as ectropic murine leukemia viruses, which interact with the adjuvant at the injection site. These antibodies could conceivably cross-react with cell surface antigens on myeloma cells.

Many antiserums which contained AIA’s were also cytotoxic for mouse myeloma cells, but there was no correlation between AIA content of an antiserum and its cytotoxicity (Chart 1). Furthermore, the cytotoxic antibodies in mouse anti-MOPC 315 IgA antiserum were active against the non-IgA-producing clone 36 variant of MOPC 315 and the IgG2-producing MOPC 31C and P3 cell lines, indicating that they were not idiotype specific (Chart 3). These observations strongly suggest that the cytotoxic antibodies in the mouse antiserum were not AIA’s. Since FA itself generates cytotoxic antibodies in mouse serum, it is likely that the cytotoxicity observed in some of the AIA-containing sera resulted from immunization with the FA portion of the immunogen rather than with the immunoglobulin. We have also found that FA was absolutely necessary for the production of AIA against MOPC 315 IgA. Recently, AIA has been shown to be produced by the administration of antigen-antibody complexes (18). Whether cytotoxicity is generated as a result of such an immunization schedule is not known.

The present studies have shown also that IgG,-producing P3 myeloma cells, which appear to be unaffected by mouse anti-FA antiserum as assessed by dye exclusion, are nevertheless prevented from secreting immunoglobulin and forming clones in agar (Chart 6). This result emphasized that such antiserum exert effects which can be detected only by using an assay system which is more sensitive than dye exclusion. Schreiber and Leibson (24) have recently shown that AIA prepared in rabbits against myeloma immunoglobulin inhibited DNA synthesis and clone formation of myeloma cells in agar. These effects of AIA depended on the presence of complement or normal spleen cells, and AIA was not cytotoxic as assessed by 51Cr release or dye exclusion. Since these workers immunized their rabbits with immunoglobulin in CFA, the possibility exists that their results were a consequence of antibody to FA rather than of AIA.

An experimental model system has been described in which either antiimmunoglobulin antibodies or AIA’s were shown to protect mice from developing myelomas or lymphomas when such tumors were transplanted after administration of these antibodies. Protection has been produced either as a result of immunization with myeloma immunoglobulin or has been passively transferred to normal hosts using immune serum (1–3, 7, 9, 11, 12, 16, 19, 20, 25, 26). The mechanism of protection was initially believed to be via a cytotoxic effect of AIA for the tumor cells. However, further studies failed to show that AIA was cytotoxic in vitro, as assessed by dye exclusion (7, 11, 12, 25), and, moreover, more than 90% of the AIA’s were of the IgG class, which is noncomplement fixing (12). Therefore, currently, it is believed that idiotype-specific transplantation resistance involves more than AIA and complement. It is unlikely that the cytotoxic antibody produced as a result of FA administration is related to in vivo myeloma and lymphoma transplantation resistance, since cytotoxicity was generated with both CFA and IFA, while transplantation resistance occurred only with immunoglobulin in CFA (2). Furthermore, transplantation resistance was idiotype specific (i.e., administration of FA together with an unrelated myeloma immunoglobulin did not produce resistance).

The antigenic component of the myeloma cells against which the FA or MDP-induced cytotoxic antibodies are directed has not been identified. We have labeled the surfaces of myeloma cells by lactoperoxidase-catalyzed iodination and have used the anti-FA antiserum to precipitate lysates prepared from these cells. However, no radioactively labeled antigenic components were identified, indicating that they were either unlabeled or present at very low concentrations. Mouse myeloma cells express a number of surface antigens, including a specific plasma cell antigen, tumor-associated transplantation antigens, oncofetal antigens, viral antigens, and immunoglobulin (22). Further studies are required to determine whether the FA-induced cytotoxic antibodies are cross-reacting with one of these components.

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* R. Bauml, A. Marks, J. Mahony, and A. Bose, unpublished observations.


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