GENERATION OF PRIMARY CYTOTOXIC LYMPHOCYTES AGAINST NON-MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGENS BY ANTI-Ia SERUM PLUS COMPLEMENT-TREATED LYMPHOCYTES*

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Histocompatibility loci have been classified as major histocompatibility complex (MHC) or minor histocompatibility complex (non-MHC) based on the time-course of in vivo graft rejection, the relative ease with which alloantiserum may be produced, and the characteristics of the in vitro mixed-leukocyte culture (MLC) (1). Attempts to sensitize cytotoxic lymphocytes (Tc) against minor histocompatibility antigens in vitro have failed (2-5). This does not seem to reflect the absence of non-MHC-antigen-recognizing Tc precursor cells; in vivo or in vitro primed lymphocytes, secondarily challenged in vitro, give rise to strong cytotoxic responses (6-8). Because the immunological outcome of antigenic challenge most probably depends upon a balance between positive and negative influences on certain fundamental pathways, we theorized that the failure to induce primary Tc in MHC-identical, non-MHC-disparate strain combinations might be a result of either a suppressive mechanism that impedes Tc development, or the absence of appropriate amplification mechanisms. We sought evidence for this hypothesis by testing cytotoxic responses of anti-Ia serum plus complement-treated lymphocytes cultured in vitro with MHC-identical, non-MHC-disparate stimulating cells. We selected this approach on the basis of evidence for differential expression of I-region determinants on regulatory T cells in other systems (9), and the strong reactivity of the antiserum used herein with T lymphocytes (10). Our results demonstrate that the responding cells that remain can mount a highly significant primary, in vitro Tc response to non-MHC antigens in several strain combinations.

Materials and Methods

Animals. B10.BR, B10.D2, and B10.S mice were bred in our colony (University of Wisconsin, Madison, Wis.). C3H/HeJ, AKR/J, BALB/c, and CBA/J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. Male and female animals 6-12 wk of age were used. Guinea pigs were obtained from Mogul Ed., Oshkosh, Wis.

Media. All media and supplements were purchased from Grand Island Biological Co., Grand Island, N. Y. Hanks' balanced salt solution without bicarbonate was supplemented with...
0.05 M Hepes buffer (HBSS). Mishell-Dutton medium (11), freshly prepared and supplemented with $5 \times 10^{-5}$ M 2-mercaptoethanol and 2% heat-inactivated fetal calf serum (FCS) (Flow Laboratories, Inc., Rockville, Md.), was used throughout. Cytotoxicity assays were performed in cell culture medium fortified with 5% FCS.

**Antisera.** (A.TH × B10.HTT)F1 anti-A.TL serum, hereafter termed anti-Ia, was prepared by concanavalin A (Con A) thymocyte blast immunization as described by Hayes and Bach (12). The method of Reif and Allen (13) was used to prepare anti-Thy-1.2 serum. Antisera were tested by a two-stage, dye-exclusion microcytotoxicity test (12). Anti-Ia serum lysed >50% of A.TL lymph node cells, with half-maximal lysis at a 1:1,280 dilution. Anti-Thy-1.2 lysed >95% of C3H/HeJ thymocytes, with half-maximal lysis at a 1:640 dilution. Fresh guinea pig serum, obtained bimonthly by heart puncture, served as complement (C). For anti-Ia serum lysis of splenocytes, cells were suspended in HBSS-diluted antiserum (1.0 ml of 1:20/5 × 10⁷ cells) and incubated on ice for 15 min. 10 vol of cold HBSS were added, the cells pelleted (1,000 rpm, 10 min), and resuspended in HBSS-diluted C (1.0 ml of 1:25/5 × 10⁷ cells). After 30 min at 37°C, the cells were washed twice with HBSS and resuspended in culture medium. Cultured lymphocytes were lysed with anti-Thy-1.2 serum plus C according to the same protocol, except that serum was diluted 1:15, C was absorbed with mouse spleen cells (5 ml of 1:5-diluted C; 1 × 10⁸ cells; 15 min on ice), and the treatment was repeated one additional time. For absorption experiments, HBSS-diluted anti-Ia serum (1.0 ml of 1:20) was absorbed twice (15 min on ice) with either B10.S or B10.BR splenocytes (1 × 10⁶ cell/absorption).

**Cell Counting.** Cell viability was determined by trypan blue exclusion staining. Cell concentrations are expressed as viable cells per milliliter.

**MLC.** Responding splenocytes, either untreated or treated with antiserum and C, were suspended in culture medium and aliquoted into microtiter wells (5 × 10⁵ cell/well) (No. 76-002-05, Linbro Chemical Co., Hamden, Conn.). X-irradiated (2,000 R), stimulating splenocytes, suspended in culture medium, were aliquoted into wells (5 × 10⁶ cell/well). Total cell culture vol was 0.2 ml. In some experiments, 24-well culture plates (No. 76-033-05, Linbro Chemical Co.) were used (5 × 10⁷ cells of each type; 2.0 ml). After 5-6 d at 37°C in a humidified atmosphere of 5% CO₂ in air, replicate cultures were resuspended with a Pasteur pipet, pooled, washed with HBSS, and resuspended in fresh medium with 5% FCS (2.5 × 10⁶ cell/ml) for cytotoxicity assay.

**Cell-mediated Lympholysis Assay.** Blast cell targets were prepared 48 h beforehand by adding Con A (2.5 μg/ml; Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) to replicate cultures of splenocytes in medium with 5% FCS (15 × 10⁶ cell/3 ml; 35 × 10-mm tissue culture dishes; No. 3001, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). To an aliquot of Con A-stimulated blast cells (5 × 10⁶ cells in 0.3 ml HBSS with 5% FCS) was added Na₂¹⁵CrO₄ (50 μl of 5 mCi/ml; New England Nuclear, Boston, Mass.), and the mixture was incubated at 37°C for 1 h, resuspending the cells at 15-min intervals. ¹⁵Cr-labeled cells, washed three times with HBSS, were resuspended in medium with 5% FCS at 5 × 10⁶ cell/ml.

³¹Cr-release assays were performed in microtiter plates (No. 76-011-05, Linbro Chemical Co.). Appropriately diluted effector cell suspensions (0.1 ml) followed by ³¹Cr-labeled target cells (0.1 ml) were dispensed into each of four replicate wells. Six replicate wells of medium and target cells served as background controls; six wells of detergent (0.5% Nonidet P40, Shell Oil Co., Houston, Tex.) and target cells gave maximal ³¹Cr-release values. Plates were centrifuged (250 g, 5 min) then incubated for 8 h at 37°C in a humidified atmosphere of CO₂ in air. A 100-μl sample was removed from each well and counted in a Packard 5320 auto gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

**Data Calculation, Presentation, and Statistical Evaluation.** Percent specific ³¹Cr release was calculated for each replicate sample according to the formula:

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\text{Percent specific } ^{31}\text{Cr release} = 100 \left( \frac{\text{experimental release (counts per minute) } - \text{ background release (counts per minute) } }{\text{maximal release (counts per minute) } - \text{ background release (counts per minute) }} \right)
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The mean value of replicate samples ± SD is presented.
Results

Primary cytotoxic responses are developed in vitro by anti-Ia serum- and C-treated B10.BR (H-2<k>) lymphocytes against H-2-identical, non-MHC-disparate stimulating cells. Untreated B10.BR cells cultured alone (▲), NMS + C-treated B10.BR cells cultured with X-irradiated CBA cells (●), and anti-Ia + C-treated B10.BR cells cultured with X-irradiated CBA cells (●) were assayed on day 6 for cytotoxic activity on ⁵¹Cr-labeled CBA target cells.

The ability to generate cytotoxicity to non-MHC antigens in a primary MLC, if the responding cells are first treated with anti-Ia serum and C, can be demonstrated in several different strain combinations (Fig. 2). In each experiment, cytotoxicity on the relevant target cell is compared for untreated, unstimulated cells; NMS and C-treated cells stimulated with X-irradiated, non-MHC-disparate cells; and anti-Ia serum and C-treated cells stimulated with X-irradiated non-MHC-disparate cells. Three combinations are shown that represent two different responding strains and two different sensitizing strains. In each case, significant cell-mediated lysis was observed only in the anti-Ia serum plus C-treated group. Lysis appeared to be antigen specific in that target cells of other strains were not lysed (C3H primed with CBA<sub>k</sub> did not lyse B10.D2 or AKR targets; B10.BR primed with AKR did not lyse CBA targets; B10.BR primed with CBA did not lyse AKR targets [data not shown]). In some instances, cross-reactivity was observed. For example, CBA-primed B10.BR cells lysed CBA (40.1 ± 11.1%) and to a lesser extent BALB/c targets (13.0 ± 9.6%) in one experiment, and CBA (67.0 ± 9.6%) and C3H targets (48.6 ± 5.2%) in a second experiment.
The cytotoxic cells that develop upon culture of anti-Ia serum plus C-treated B10.BR responding lymphocytes with CBA stimulating cells bear the Thy-1.2 antigen. In vitro primed cytotoxic effector cells, derived from cultures of anti-Ia serum and C-treated responding cells, were tested for their susceptibility to anti-Thy-1.2 serum and C lysis immediately before the cell-mediated lympholysis assay: untreated cytotoxic cells lysed CBA targets (22.6 ± 4.9%) as did NMS and C-treated cells (20.5 ± 4.9%). Two-cycle anti-Thy-1.2 plus C treatment virtually eliminated cytotoxicity (3.4 ± 3.1%). Thus, the cytotoxic effector cells appear to be T cells.

The anti-Ia serum used in these experiments has been extensively characterized with regard to its haplotype specificity (10). Absorption results confirmed this specificity: B10.BR cells that remained after treatment with unabsorbed anti-Ia serum and C lysed CBA targets (29.0 ± 8.7%) after being cultured 6 d in vitro with CBA stimulating cells. When anti-Ia serum was first absorbed with B10.BR or C3H spleen cells, Tc did not develop (−8.7 ± 7.3%). On the other hand, anti-Ia serum retained its reactivity after absorption with B10.S spleen cells (28.0 ± 11.1%).

Discussion

The results presented in this paper demonstrate that responding cells that remain after anti-Ia serum and C treatment generate a highly significant cytotoxic response against non-MH(2 antigens: NMS and C-treated control cells do not. The cytotoxic response appears to be antigen specific in that target cells of strains other than the sensitizing strain are not lysed, or lysed to a lesser extent. The cytotoxic cells are susceptible to anti-Thy-1.2 and C lysis. Thus, they appear to be Tc.

The anti-Ia serum employed in these studies has been well characterized with respect to its specificity (10). It contains antibody to both I-Ak- and I-Jk-encoded antigens; I-Bk, Qa, and Tla activity were not observed (10). Experiments are in progress to determine which antiserum component(s) are necessary to bring about non-MH(2-Tc induction.

Since the advent of transplantation, immunogeneticists have questioned the basis of phenomenological distinctions between major and minor transplantation antigens. One hypothesis to explain some characteristic immunological differences between these antigens suggests that Tc development is subject to suppressive and enhancing regulatory influences; strong and weak antigens might then differ primarily in the overall balance between such regulatory mechanisms that they elicit and, therefore, in their ability to induce Tc. The results presented here can be interpreted in view of this hypothesis by suggesting that anti-Ia serum plus C might remove from the responding cell population a cellular component of a non-MH(2-antigen-activated suppressive mechanism, permitting unimpeded non-MH(2-Tc generation in antigen-stimulated cultures (S. M. Macphail and F. H. Bach. Cytotoxicity and suppression induced by in vitro responses to non-H-2 histocompatibility antigens. Manuscript in preparation.). This interpretation is strengthened by two lines of experimental evidence. First, I-J-antigen-bearing suppressor T cells have been demonstrated in several systems (14, 15). Second, lymphocytes from non-MH(2-antigen-primed donors produce an MLC proliferation-suppressing factor (16) and block the differentiation of MHC-specific memory Tc when these are restimulated with antigen in vitro (17). If this interpretation is correct, and we regard it as a likely mechanism, this would be the first demonstration of both suppressor cells in the cell-mediated immune response...
(T_c) to non-MHC-antigens, and the existance of Ia-antigen-bearing, unprimed suppressor cells in vitro.

Other mechanisms by which anti-Ia serum and C may function cannot be ruled out. For instance, anti-Ia antibody may activate a dormant helper cell, or deliver a second requisite signal to a non-MHC-specific T_c precursor. Further experimentation should permit us to distinguish which of these possibilities is correct.

Summary

The results presented in this paper demonstrate that responding cells that remain after anti-Ia serum plus complement (C) treatment generate a highly significant in vitro cytotoxic response against minor histocompatibility complex antigens. The cytotoxic response appears to be antigen specific in that target cells of strains other than the sensitizing strain are not lysed, or lysed to a lesser extent. The cytotoxic cells are susceptible to anti-Thy-1 plus C lysis. Anti-Ia serum may function by removing an unprimed suppressor cell, although other mechanisms cannot be ruled out.

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