CONTINUOUSLY PROLIFERATING ALLOSPECIFIC T CELLS

I. Specificity of Cooperation with Allogeneic B Cells in the Humoral Antibody Response to Sheep Erythrocytes*

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It is now an established fact that thymus-derived peripheral lymphoid cells exhibit a marked heterogeneity with regard to their functions (reviewed in reference 1). This heterogeneity reflects the immunological repertoire of distinct subpopulations of T lymphocytes, lymphocytes distinguished by their physical and serological properties. Recent advances in studying T-cell function have concentrated on the enrichment of antigen-specific effector cells. At the present time, there exist several techniques for such a selective fractionation. One method involves the selection by immunological function of the cloned fusion products derived from somatic crosses between specific primed T cells (helper, cytotoxic) and established cell lines (2). A second method of clonal isolation involves the positive selection of reactive T cells by repeated stimulation with allogeneic cells (3, 4) or concanavalin A (Con A) supernates (5), followed by cloning in limiting dilution or in soft agar. Only those cells undergoing cell division would be selected for, leaving nonresponding cells to eventually die out.

Utilizing a positive selection technique, it has been demonstrated that T cells derived from mixed lymphocyte culture can be maintained in tissue culture for several years, as long as these cells are repeatedly stimulated with the alloantigens used initially (6). Noncloned cell lines derived by such procedures exhibit specific proliferative activity (6) and in some instances retain specific cytotoxic function.[1]

Recently, it has been shown that products of the major histocompatibility complex (MHC)[2] can also activate strong helper and suppressor T-cell activities (7, 8). It was of interest, therefore, to test these cell lines for their ability to cooperate in vitro with B lymphocytes in induction of a humoral response to sheep erythrocytes (SRBC).

Materials and Methods

Animals. BALB/c ($H\text{-}2^d$) were purchased from the Leonel C. Strong Research Foundation, San Diego, Calif. The B10.Br, B10.A, B10.AQR, B10.A(4R), B10.A(5R), B10.G, B10.D2, and

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[1] Dennert, G. 1978. Cytolytic and proliferative activity of permanent T killer cell line. *Nature* (*Lond.*). Submitted for publication.

[2] *Abbreviations used in this paper:* AEF, allogeneic effect factor; MHC, major histocompatibility complex; RAMB, rabbit anti-mouse brain serum; SRBC, sheep erythrocytes.

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B10 strains were bred in our colony at the Department of Biology, University of California, San Diego. Animals were age and sex matched for each experiment.

Cell Lines. C.C3.11.75[3] was established from a primary mixed lymphocyte culture containing 10^5 per ml responder BALB/c (H-2^d) spleen cells and 10^5 per ml 1,000 rads irradiated C3H (H-2^k) stimulator cells (6). Cultures were set up in 20-ml Falcon tissue culture bottles (No. 3013) (Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.) in Click's minimal essential medium supplemented with 5% selected fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 0.2 mM glutamine, 100 IU penicillin/ml, and 100 μg streptomycin/ml. Culture flasks were incubated in an upright position in 5% CO_2 in air at 37°C. Every 2 wk the culture supernate was replaced with new medium containing 10^5 per ml irradiated C3H spleen cells. The cultures were regularly split when the cell density reached 5×10^5 cells per ml. Cells used for studies on helper function were harvested 6 d after addition of irradiated C3H stimulator cells and purified on a bovine serum albumin (BSA) gradient (6).

Antisera and Complement. Spleen cells were depleted of T lymphocytes by treating sequentially with a 1/20 dilution of rabbit anti-mouse brain (RAMB) and 1/40 dilution of absorbed guinea pig complement. The procedure and properties of the RAMB serum have been described in detail previously (9).

Cell Preparations. T lymphocytes were isolated from BALB/c mouse spleen as described by Julius et al. (10). Briefly, 1 ml of a spleen suspension, 70×10^6 cells/ml in complete RPMI medium (RPMI-1640 plus 0.2 mM glutamine, 100 IU penicillin/ml, 100 μg streptomycin/ml, 15 mM Hepes buffer, 5% heat-inactivated fetal calf serum (FCS), and 5×10^{-5} M 2 Me) (Media Preparation Unit, University of California at San Diego) was layered on a 10-ml syringe containing 0.6 g acid-detoxified nylon wool previously equilibrated with complete RPMI medium. The columns were then incubated at 37°C for 30 min and the cell eluted. These T cells, together with the C.C3.11.75 cells, were treated with 25 μg mitomycin C for 20 min and washed three times before use in the assay system. Such treatment prevents induction of suppressor and/or cytotoxic activity (8). B. lymphocytes were prepared from the relevant strains as described above.

Allogeneic Supernates. 1×10^6 C.C3.11.75 mitomycin C-treated T lymphocytes were cultured together with 1×10^7 mitomycin C-treated splenic B lymphocytes (RAMB + C) from B10.D2 and B10.Br animals. The cultures were carried out in a 5-ml final volume in complete RPMI. After 24 h the supernates were harvested by centrifugation (10 min at 1,500 *g* to remove the cells), aliquoted, and frozen at −20°C until use.

Positive Allogeneic Effect Assay. The positive allogeneic effect was evaluated in a primary anti-SRBC response in vitro (11). T-cell preparations were added in varying numbers to a constant number of B cells ($5.0–8.0 \times 10^5$/well), all preparations resuspended in complete RPMI. Cell cultures were carried out in Falcon 3040 Microtest II plates (Falcon Plastics) in a 0.1-ml final volume. Sheep erythrocytes (0.1% vol/vol in balanced salt solution [BSS]) were added to each well in a 10-μl volume. The cultures were placed in plastic boxes and gassed with a 10% CO_2, 7% O_2, and 83% N_2 mixture. The cells were fed with 1/10 volume complete cocktail and gassed daily. Day 4 cultures (optimal plaque-forming cells [PFC] response) were assayed for direct antibody-forming cells by hemolysis in agar, modified from the method of Jerne (12). Data points for each titration value are the geometric means ± standard deviation of triplicate wells.

Results

Demonstration of a Humoral Anti-Sheep Erythrocyte (SRBC) Response Activated by the Allospecific T-Cell Line C.C3.11.75. Allospecific T cells were positively selected and maintained in tissue culture for ≅3 yr by repeated alloantigenic simulation. Such cells, although noncloned, hava been shown to exhibit restricted immunological specificity in both the proliferative and cytotoxic assays (6).[1] It was of interest, therefore, to determine whether these cells could exhibit helper function (positive allogeneic effect) in induction of a humoral response to SRBC and if so, whether such

[3] This nomenclature designates a BALB/c (H-2^d) anti C3H (H-2^k) noncloned cell line established in November 1975.

helper function displayed specificity for the alloantigens used in selection. Accordingly, C.C3,11.75 T lymphocytes and normal BALB/c T lymphocytes (both groups mitomycin-C treated) were added in varying numbers to B lymphocytes from B10.Br (H-2^k), B10 (H-2^b), and B10.D2 (H-2^d) mice. These mice were chosen for evaluation of a potential allogeneic effect as they differ from each other at the chromosome region carrying the MHC, thereby reducing possible background genetic influences, and, they share the same Mls type as BALB/c. It can be seen from Fig. 1 A that both the BALB/c (H-2^d) and the C.C3.11.75 (H-2^d) T lymphocytes induced a positive allogeneic effect with the B10.Br (H-2^k) B lymphocytes. The individual titration curves, however, differed between the two T-cell preparations. The C.C3.11.75 cells displayed an 81-fold enrichment of helper function. Significant suppression was seen with cell numbers that, using BALB/c T cells, elicit optimal allohelp. When both groups of T cells were tested on B10 (H-2^b) B lymphocytes (Fig. 1 B), the C.C3.11.75 cells failed to give significant allohelp, demonstrating the specificity of the cell line for the alloantigenic determinants used in their selection. Finally, in Fig. 1 C (a specificity control) no positive allogeneic effect was seen when both groups of T cells were tested on B cells expressing the syngeneic MHC (H-2^d) haplotype.

Intra-H-2 Mapping of the Gene(s) Coding for Antigens Required in Induction of the Positive Allogeneic Effect. Because C.C3.11.75 T cells are able to exert helper function on B cells expressing the H-2^k antigens, we investigated which subregion(s) in the H-2^k MHC is important for induction of allohelp. For this purpose, a series of intra-H-2 recombinant mice (again, all on the B10 background) were used as donors of the B lymphocytes. The experiments were set up as described in the previous section, titrating BALB/c and C.C3.11.75 T cells against a constant number of B lymphocytes. The data were evaluated as follows. C.C3.11.75 cells reproducibly showed a 27-fold enrichment of effector function (on a per cell basis) when compared with the response of BALB/c T cells. The numbers of plaque-forming B lymphocytes from the different intra-H-2 recombinant strains were compared at the peak point. The results of a representative experiment are shown in Fig .2. The C.C3.11.75 T cells activated significant antibody synthesis in B10.Br, B10.A, B10.AQR, and B10.A(4R) B cells, the B10.A(4R) giving as high a response as B10.Br (whole haplotype). B10.A(5R) B cells, expressing IJ^k- and IE^k-region homology with the stimulating haplotype, exhibit a significantly lower level of antibody synthesis. Finally, B10.G, B10, and B10.D2 B cells display no response, again verifying the specificity of the C.C3.11.75 cell line for H-2^k. These results suggest that the IA subregion codes for the alloantigens recognized in the induction of allohelp, but do not exclude contributions from the K and/or IB regions, and perhaps, to a lesser extent, IE and IJ.

Specificity of an Allogeneic Effect Factor Derived from the C.C3.11.75 T-Cell Line. Previous findings have indicated that the allogeneic effect factor(s) may display a limited specificity, preferentially activating B lymphocytes syngeneic to the stimulator and responder cells (13). In the past two sections we have demonstrated that the C.C3.11.75 cell only induces specific helper function with B cells sharing H-2^k subregion(s) with the strain used for stimulation. We tested whether an allogeneic effect factor is secreted by confrontation of this cell line with mitomycin c-treated B10.Br B lymphocytes, and if so, whether this factor exhibits strain-specific helper activity in the primary humoral response to sheep erythrocytes. In Fig. 3 we demonstrate that a helper factor is released upon stimulation with B10.Br but not by stimulation with

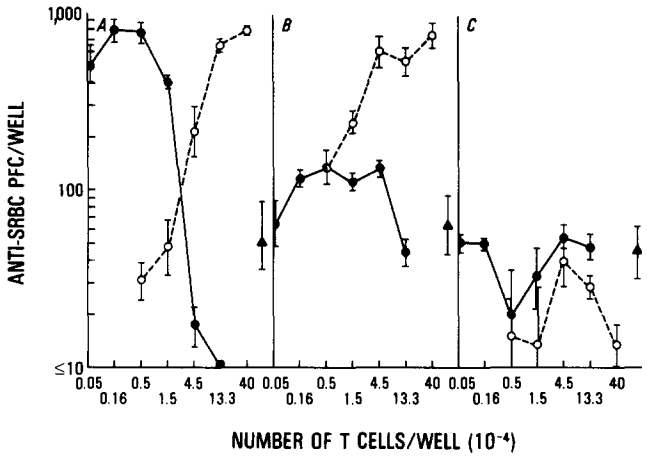

FIG. 1. Varying numbers of nylon-wool column passed T cells from BALB/c (○) and the C.C3.11.75 cell line (●), both mitomycin C treated, were added to wells containing SRBC and 5 × 10^5 B lymphocytes from: (*A*) B10.Br mice; (*B*) B10 mice; and (*C*) B10.D2 mice. PFC responses were determined on day 4. The data points represent the geometric mean ± standard deviation of triplicate wells. Background responses of B cells alone are indicated (▲). The figure represents one of four separate experiments.

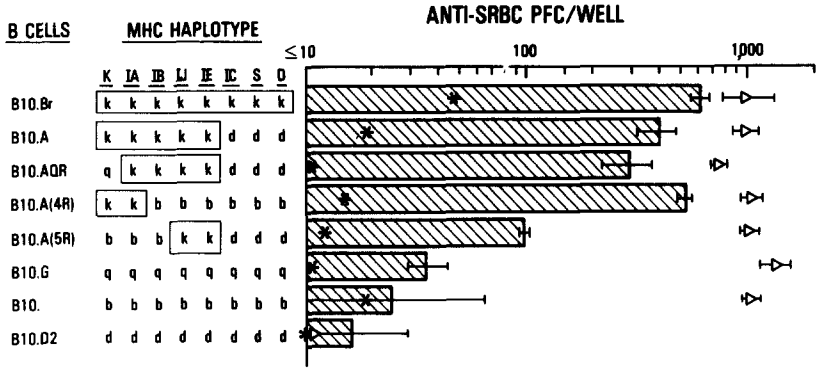

FIG. 2. Varying numbers of nylon wool column passed T cells from BALB/c and C.C3.11.75 cell line, both mitomycin C treated, were added to wells containing SRBC and 5 × 10^5 B lymphocytes from the listed recombinant inbred mice. The results represent the geometric mean ± standard deviation of PFC activated by 1.5 × 10^4 C.C3.11.75 T lymphocytes (peak antibody response) (□). Background responses of B cells alone are indicated (*). Normal BALB/c T lymphocytes routinely activated all the B cells listed except B10.D2 as would be expected (△). However, the peak response occurred at a 27-fold higher concentration using these cells. The figure represents one of three separate experiments.

B10.D2 B cells. It can also be seen that the factor nonselectively activated B lymphocytes from B10.A(4R), B10, and B10.D2 mice and therefore can be considered nonspecific.

Discussion

A number of studies have demonstrated the ability of allogeneic lymphocytes to activate humoral antibody synthesis in vitro (9, 11, 14, 15). Earlier workers found that the primary immune SRBC response of cells from either adult thymectomized,

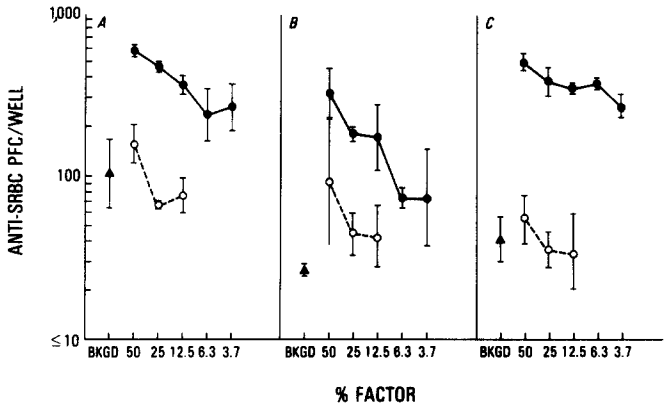

FIG. 3. Varying concentrations of allogeneic effect factor, derived from admixed C.C3.11.75 cells and either 10^7 B10.D2 (O- - -O) or B10.Br (●——●) mytomycin C B lymphocytes were added to wells containing SRBC and 5 × 10^5 B lymphocytes from: (*A*) B10.A(4R) mice; (*B*) B10 mice; and (*C*) B10.D2 mice. PFC responses were determined on day 4. The data points represent the geometric mean ± standard deviation of triplicate wells. Background responses of B cells alone are indicated (▲). The figure represents one of three separate experiments.

irradiated, bone-marrow protected mice (14) or neonatally thymectomized mice (7) could be restored by the addition of allogeneic spleen cells to the in vitro culture. Lefkovits and coworkers extended these observations by showing, first, a θ-sensitive allogeneic T cell was required for eliciting the anti-SRBC response (16) and second, using limiting dilution analysis, specific alloantigen-reactive T cells mediated this response (17). In studying the specificity and mechanism of action, Kettman and Skarvall, measuring responses of third-party B lymphocytes (bystander), confirmed that histocompatible differences were required for triggering and demonstrated that the response could be mediated through release of soluble factors (18). Subsequent work on such factors has indicated that, depending on the in vitro system used, the factors act either nonspecifically (in terms of H-2 haplotype [19]) or exhibit a limited strain specificity (13, 20).

The cell line C.C3.11.75, propagated in tissue culture for almost 3 yr by repeated allostimulation, has been shown to exhibit specific proliferative and cytotoxic functions. The stimulating determinants and target antigens for lympholysis appear to be coded for by genes located in the IA subregion of the mouse MHC.[1] In this communication we report that C.C3.11.75 T lymphocytes can also exert helper function during the induction of a humoral response to SRBC. The help was specific in that the B cells activated had to share MHC antigens with the strain used for selection of the cell line. Intra H-2 mapping showed that genes in the IA subregion appeared to be important for the induction of the positive allogeneic effect. Thus, the induction of help is a result of recognition of the H-2 subregion that is also responsible for proliferation. Supernatant factor(s) could substitute for the T cells in activation of the in vitro humoral response. Although this factor(s) exhibited no strain specificity in its action, its secretion was dependent on allorecognition. It appears that the specificity seen in the positive allogeneic effect is a consequence of the alloantigenic recognition receptors intrinsic to the cell line, and not to any biologically restricting properties of the allogeneic effect factor itself.

These findings raise the issue of whether the supernatant factor(s) responsible for augmenting the B-cell response in these cultures is conventional allogeneic effect

factor (AEF), which might be expected to exhibit limited specificity (13) or if it is another T-cell mediator. Clearly, our data indicate that if it is AEF, it exhibits no strain specificity. Furthermore, like AEF, factor production is not dependent on the proliferation of the T cells (13). Strict classification of the factor by mechanism of action has not yet been carried out. It is possible that this factor is not a true T-cell replacing factor, like AEF, acting directly on the B-cell population. The factor may prove to act by promoting residual T cells left after RAMB + C treatment. An analysis of splenic lymphocytes from nude mice will hopefully resolve this issue.

Demonstration of helper activity in a selected T-cell line contradicts an earlier finding by one of us which failed to show similar helper activity (21). The discrepancy, however, can be explained by differences in the experimental system used. The cell line C.C3.11.75 was not tested in the previous report. It is possible that during the selection of cells by proliferation the C.C3.11.75 line maintained its helper activity, whereas in the other lines tested helper activity was selected against. Furthermore, the cell line was tested 6 d after stimulation with 1,000 rads irradiated C3H stimulator cells. In the previous report the allospecific T cells were used 3 d after restimulation. The kinetics of helper function in the in vitro primary immune response appear to be critical. The maximum activity was seen when the cells tested were in a refractory state, suppressive effects being observed when C.C3.11.75 was tested 3 d after stimulation (J. D. Waterfield, unpublished observations).

These findings raise certain questions regarding the homogeneity of the cell line. It is possible that more than one subset of T lymphocyte has been selected for. Conversely, one would have to propose that the cells of the allospecific cell line are capable of performing more than one function. Experiments utilizing anti-Ly sera, limiting dilution analysis, and cloning of C.C3.11.75 are in progress to distinguish between these possibilities.

In conclusion, these findings, together with previous work, demonstrate that a positive selection procedure, based on the ability of cells to proliferate to an alloantigenic stimulus, selects for subsets of T cells exhibiting different immunological function. Thus, the C.C3.11.75 cell line contains lymphocytes reactive in MLR, CML, and helper cell responses; the effector cells displaying specificity to the I-A subregion of the selecting MHC.

Summary

Allospecific mouse T cells, positively selected in one-way mixed lymphocyte culture were maintained for 3 yr in tissue culture by sequential restimulation. Such proliferating T cells were tested for their ability to induce a positive allogeneic effect: activating B cells in an in vitro primary humoral response to sheep erythrocytes. It was found that such T lymphocytes could function as helper cells. Helper activity was shown to be specific in that the B cells activated had to share major histocompatibility complex (H-2) antigens with the strain used for selection of the cell line. Intra H-2 mapping showed that antigens coded in the IA^k subregion played an important role in the induction of the positive allogeneic effect. Supernatant factors could substitute for the allogeneic T cells in activation of the in vitro humoral response. However, such supernates exhibited no strain specificity. Therefore, the specificity seen in the positive allogeneic effect is presumably a consequence of the alloantigenic recognition receptors intrinsic to the T cells, and not to any biologically restricting properties of the allogeneic effect factor itself.

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