ROLE OF *I*-REGION GENE PRODUCTS IN MACROPHAGE INDUCTION OF AN ANTIBODY RESPONSE

II. Restriction at the Level of T Cell in Recognition of *I-J*-Subregion Macrophage Determinants*

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Genetically restricted macrophage-T cell interactions during the induction of an immune response have been described by a number of investigators (1–15).[1] Rosenthal and Shevach (1) first demonstrated that proliferation of primed guinea pig T cells required the presence of syngeneic macrophages. It followed that the in vitro murine response to soluble antigens also required that macrophages and T cells be compatible at the *I* region (3). Pierce et al. (7) observed that the random terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT)^2-primed T lymphocytes only responded in vitro to macrophages bearing the same *H-2* products as the macrophages used to prime the T cells. No such restriction was found, however, in the original priming of the T cells. F_1 chimeric mice reconstituted with stem cells of both parental strains provided Erb et al. (11) with mutually tolerant T lymphocytes of the two parental haplotypes. In their experiments, T cells of one haplotype were not able to function with allogeneic macrophages, which provided evidence that the genetic restrictions imposed on the macrophage-T cell interaction were not the result of antigen priming but reflected the differentiation of the T cell.

Although there has been some disparity in experimental observations by the various research groups, there exists a unifying hypothesis that the T amplifier cells not only recognize antigenic determinants of T-dependent antigens but must also recognize a product of the major histocompatibility complex *I* region (1, 2, 4, 9–15).[1] The nature of the T cell recognition unit, the structure of the macrophage *I*-region determinants, and the relationship between the genetic restriction of the induction of a response and Ir genes is still not known.

In previous reports we have detailed experiments that indicated that the recognition unit on the macrophage was a product of the *Ia-4* locus and that the blocking of this

determinant with antibodies specific for products of the *I-J* subregion inhibited the macrophage-T cell interaction in primary and secondary in vitro antibody responses to burro erythrocytes (BRBC) (13, 14, 16, 17).[1] In this manuscript, experiments with F_1 hybrids of strains that differ only at the *Ia-4* locus demonstrate the specificity of anti-*I-J* sera blocking of the macrophage-T cell interaction and indicate the presence of a T cell receptor for this determinant.

Materials and Methods

Mice. The experiments were conducted with B10.A(3R) (H-2^{i3}), B10.A(5R) (H-2^{i5}), B10.S(9R) (H-2^{t4}), B10.HTT (H-2^{t3}), and F_1 hybrids [B10.A(3R) × B10.A(5R)]F_1 and [B10.S(9R) × B10.HTT]F_1. Mice in each experiment were matched for age and sex. The original breeders for this colony were obtained from The Jackson Laboratory, Bar Harbor, Maine or Dr. Donald Shreffler, Washington University, St. Louis, Mo.

Antisera. Restricted anti-Ia alloantisera were prepared as previously described (17–20). Batches of sera from several bleedings after a series of immunizations were tested in a dye exclusion microcytotoxic assay for appropriate anti-Ia reactivity and antibody titer. The sera prepared to react only with determinants of the *I-J* subregion did not demonstrate any lytic activity when tested on lymph node cells and spleen cells in the microcytotoxic test. Adsorption of sera was performed at 37°C for 45 min followed by a 15-min incubation at 4°C with 10^8–10^9 washed lymphoid cells/ml of neat serum. Adsorbed sera were tested in a dye exclusion microcytotoxic assay against appropriate targets.

Batches of anti-Thy-1.2 sera were prepared by immunizing A.AKR (H-2^{al}, *Thy-1^a*) mice with A.AL (H-2^{al}, *Thy-1^b*) thymocytes. Rabbit anti-mouse IgG serum was prepared by injecting rabbits with rabbit erythrocytes coated with mouse anit-rabbit erythrocyte hyperimmune antibodies.

Complement for antisera testing and experiments was obtained by cardiac puncture from 2- to 4-wk-old rabbits. The complement was screened against thymocytes and lymph node cells for natural cytotoxicity, and only batches with <5% natural cytotoxicity were used.

Culture Conditions. Dispersed spleen cell cultures were prepared according to the method of Mishell and Dutton (21). The cultures were established in 35-mm plastic Petri dishes (No. 3001, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) with each dish receiving a daily addition of 90 µl of enriched culture media. Cultures were immunized with 10^7 BRBC (Colorado Serum Co., Denver, Colo.) and maintained for 5 d at 37°C in an atmosphere of 10% CO_2, 7% O_2, and 83% N_2 while rocking at 7 cycles/min.

Macrophage isolation from spleen cells and the preparation of the macrophage-depleted T-B cell population has been described in previous publications (17).[1] In the experiments reported here, purified macrophage monolayers were incubated with anti-Ia serum diluted 1:10 for 30 min then washed by adding fresh culture medium and decanting two times. The macrophage monolayers were not treated with complement but were further blocked with mitomycin-C (Aldrich Chemical Co., Inc., Milwaukee, Wis.) for 30 min at 37°C (40 µg/10^7 cells per ml). The monolayers were again washed four times by the repeated addition of fresh medium. Macrophage-depleted T-B cells obtained from normal mice were added to each macrophage monolayer (10^7 cells/ml per dish), and the cultures were stimulated with the antigen (10^7 BRBC).

The primary IgM response was assayed at the end of the culture period by determining the number of direct plaque-forming cells (PFC) (22, 23). Each culture dish was individually assayed, and the mean and standard deviation of quadruplicate dishes were determined.

Macrophage Isolation. Adherent splenic phagocytic cells were prepared as previously described (17).[1] Briefly, dispersed spleen cells were seeded into plastic Petri dishes at a concentration of 10^7/ml and incubated for 2.5 h. Nonadherent cells were decanted by two additions of fresh medium. Anti-Thy-1.2 serum (1:10 final dilution) and rabbit anti-mouse IgG serum (1:100 final dilution) were added to the dishes, and the dishes were returned to the 37°C chamber. After a 30-min incubation with gentle rocking, the antibodies were decanted, rabbit complement was added, and the incubation continued for 30 min. The macrophages were again washed by adding fresh medium and decanting. The number of adherent cells remaining in each dish ranged from 0.5 to 1.2 × 10^6 cells.

Macrophage-depleted Cell Fraction.   Spleen cells were incubated for 40 min at 37°C with carbonyl iron powder (Atomergic Chemetals Corp., Plainview, N. Y.) (17).[1] The iron powder and phagocytic cells were removed with a strong magnet, and the carbonyl iron treatment was repeated a second time. Cells that incorporated latex particles and that had morphologic characteristics of macrophages comprised <1% of this remaining cell population. Total cell loss with this procedure was 40–50%, but no difference in the T cell:B cell ratio could be discerned.

Results

Specificity of Anti-I-J Sera Blocking of F_1 Macrophages.   As previously reported, only antibodies raised against an incompatible *I-J* subregion could effectively block the function of antigen-presenting macrophages when these macrophages were subsequently cultured with normal T cells and B cells (13, 14).[1] Anti-Ia reagents restricted to detect determinants of subregions *I-A*, *I-E*, or *I-C* always required the presence of complement to actually delete the Ia^+ macrophages.[1] These observations led to experiments with splenic macrophages purified from [B10.A(3R) × B10.A(5R)]F_1 hybrids and T-B cells depleted of macrophages prepared from syngeneic F_1 or parental mice. The B10.A(5R) and the B10.A(3R) strains differ only at the *I-J* subregion. The experiment in Fig. 1 demonstrates that the F_1 macrophages can support the primary antibody response to BRBC of F_1 or parental strain T-B cells.

If the F_1 macrophages were first blocked with anti-*I-J^k* antibodies, they could support the response of F_1 T-B cells and B10.A(3R) T-B cells but not B10.A(5R) T-B cells. The F_1 macrophages treated with anti-I-J^b serum could support the response of F_1 T-B cells and B10.A(5R) T-B cells but not B10.A(3R) T-B cells. A second identical experiment is shown in Fig. 2.

The experiment was repeated with a different strain combination differing at the *I-J* subregion, [B10.S(9R) × B10.HTT]F_1 mice. The F_1 splenic macrophages were blocked with anti-*I-J^k* serum and combined with F_1 T-B cells or with parental T-B cells (Figs. 3 and 4). Antibodies reactive with *I-J^k*-subregion determinants on these macrophages did not block the response when F_1 T-B cells were used with B10.HTT T-B cells. The response was significantly reduced when *I-J^k* blocked macrophages were used with B10.S(9R) T-B cells. In the second portion of each experiment the macrophages were blocked with antibodies directed at *I-J^s*-subregion determinants. The F_1 *I-J^s*-blocked macrophages worked with F_1 T-B cells and with B10.S(9R) T-B cells but not with B10.HTT T-B cells.

These experiments indicated that the anti-*I-J* serum-blocked F_1 antigen-presenting macrophage could only be recognized by the $Lyt-1^+,2^-,3^-$ T helper (T_H) cell bearing a receptor syngeneic for the unblocked *I-J* subregion. Such experiments also provide further poof that the inhibition of the response is a specific interference with the macrophage-T cell interaction and not the result of the induction of suppression.

Anti-I-J Serum Treatment of Parental Macrophages.   The specificity of the anti-*I-J* serum blocking of macrophage recognition by the $Lyt-1^+,2^-,3^-$ syngeneic T_H cell was further demonstrated when macrophages were purified from either parental strain. In the experiment shown in Fig. 5, the addition of [B10.A(3R) × B10.A(5R)]F_1 T-B cells to parental macrophages resulted in a PFC response essentially equal to the controls that consisted of B10.A(3R) T-B cells added to B10.A(3R) macrophages and B10.A(5R) T-B cells added to B10.A(5R) macrophages. If, however, B10.A(3R) macrophages were pretreated with anti-*I-J^b* serum, washed in fresh medium, and then cultured with F_1 T-B cells, the PFC response was significantly inhibited. As expected,
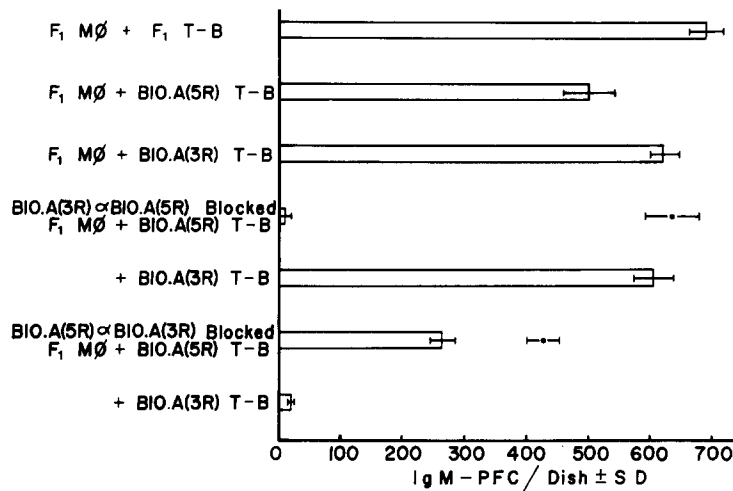
Fig. 1. Effect of anti-*I-J* serum-blocking treatment of (J^k × J^b)F1 macrophages on the subsequent interaction of these macrophages with F1 or parental T-B cells. Antisera B10.A(3R) anti-B10.A(5R) (anti-J^k) and B10.A(5R) anti-B10.A(3R) (anti-J^b) were used without complement to pretreat F1 macrophages before culturing with T-B cells and antigen. Antiserum [A.TH × B10.A(5R)] anti-A.TL was used as an inappropriate anti-Ia serum in this experiment with no blocking effect on the F1 macrophages [406 ± 33 PFC with B10.A(3R) T-B cells and 440 ± 22 PFC with B10.A(5R) T-B cells]. The symbol ● represents the PFC response with F1 T-B cells. Each bar represents the mean ± SD of IgM-PFC response of quadruplicate dishes on day 5 of culture.
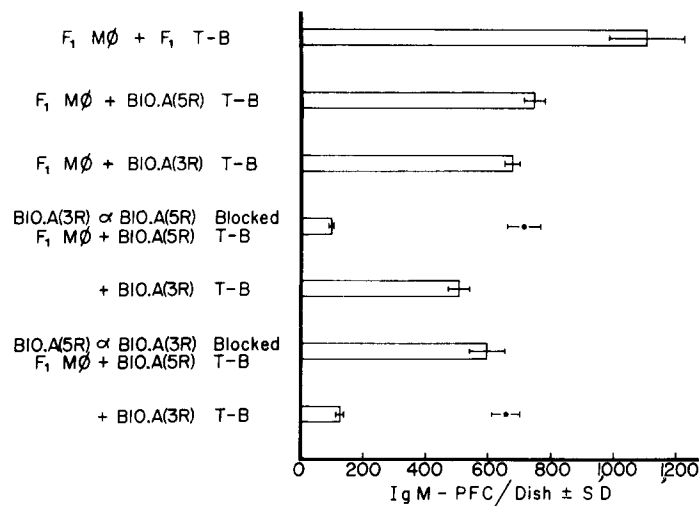

Fig. 2. Effect of anti-*I-J* serum-blocking treatment of (J^k × J^b)F1 macrophages on the subsequent interaction of these macrophages with F1 or parental T-B cells. Antisera B10.A(3R) anti-B10.A(5R) (anti-J^k) and B10.A(5R) anti-B10.A(3R) (anti-J^b) were used without complement to pretreat F1 macrophages before culturing with T-B cells and antigen. The symbol ● represents the PFC response of blocked macrophages cultured with F1 T-B cells. Each bar represents the mean ± SD of quadruplicate dishes on day 5 of culture.
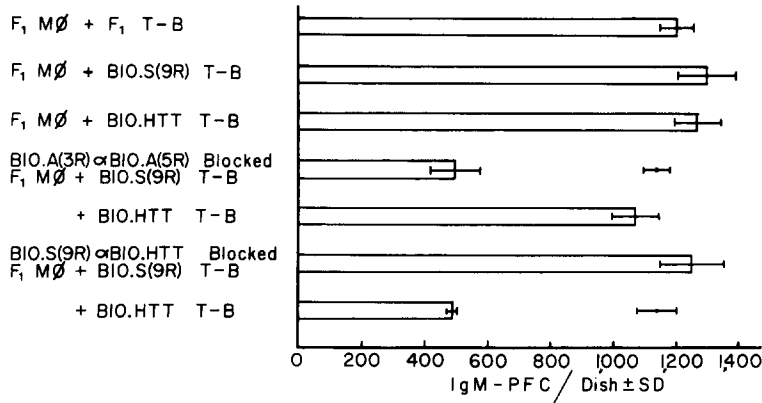
Fig. 3. Effect of anti-*I-J* serum-blocking treatment of (J^k × J^s)F$_1$ macrophages on the subsequent interaction of these macrophages with F$_1$ or parental T-B cells. Antisera B10.A(3R) anti-B10.A(5R) (anti-J^k) and B10.S(9R) anti-B10.HTT adsorbed with *H-2^k* cells (anti-J^s) were used without complement to pretreat F$_1$ macrophages before culturing with T-B cells and antigen. F$_1$ macrophages blocked with an inappropriate serum [(B10.A × B10.D2)] anti-B10.A(5R) gave normal responses of 1,020 ± 98 PFC with B10.S(9R) T-B cells and 1,193 ± 5 PFC with B10.HTT T-B cells. The symbol ● represents the PFC response of blocked macrophages cultured with F$_1$ T-B cells. Data is presented as the mean ± SD of quadruplicate dishes on day 5 of culture.

Fig. 4. Effect of anti-*I-J* serum-blocking treatment of (J^k × J^s)F$_1$ macrophages on the subsequent interaction of these macrophages with F$_1$ or parental T-B cells. Antisera B10.A(3R) anti-B10.A(5R) (anti-J^k) and B10.S(9R) anti-B10.HTT adsorbed with *H-2^k* cells (anti-J^s) were used without complement to pretreat F$_1$ macrophages before culturing with T-B cells and antigen. In this experiment, F$_1$ macrophages were also blocked with B10.S(7R) anti-B10.HTT serum adsorbed with B10.A(4R) and B10.D2 cells (anti-E^k). Anti-E^k-blocked macrophages responded normally with B10.S(9R) T-B cells (842 ± 20 PFC) and with B10.HTT T-B cells (739 ± 100 PFC). F$_1$ macrophages blocked with an inappropriate serum [(B10.A × B10.D2)] anti-B10.A(5R) gave normal responses of 898 ± 40 PFC with B10.S(9R) T-B cells and 890 ± 27 PFC with B10.HTT T-B cells. The symbol ● represents the PFC response of blocked macrophages cultured with F$_1$ T-B cells. Data is presented as the mean ± SD of quadruplicate dishes on day 5 of culture.
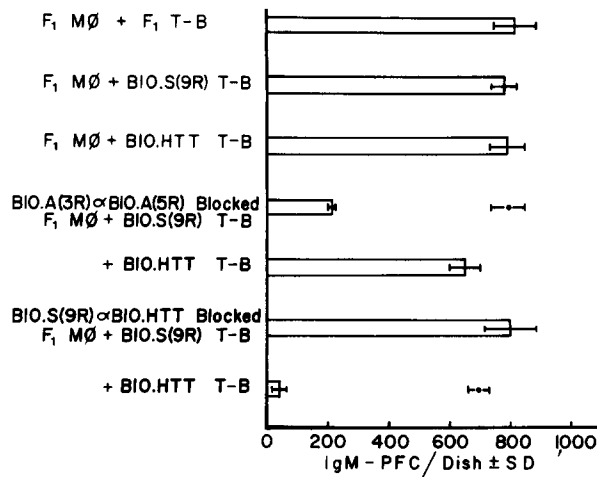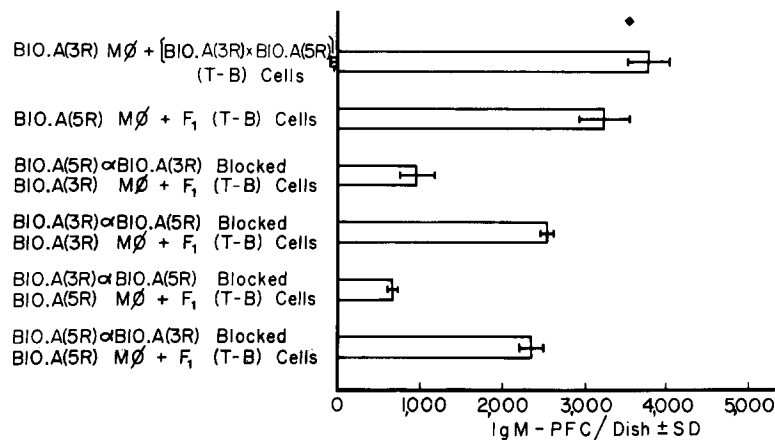
Fig. 5. Effect of anti-*I-J* serum-blocking treatment of J^k or J^b parental macrophages on the subsequent interaction of these macrophages with F_1 T-B cells. Antisera B10.A(3R) anti-B10.A(5R) (anti-J^k) and B10.A(5R) anti-B10.A(3R) (anti-J^b) were used without complement to pretreat macrophages before culturing with F_1 T-B cells and antigen. The symbol ◆ represents the response when culturing B10.A(5R) macrophages with B10.A(5R) T-B cells and the symbol ◇ represents the response when culturing B10.A(3R) macrophages with B10.A(3R) T-B cells. Data is presented as the mean ± SD of quadruplicate dishes on day 5 of culture.

treatment of the B10.A(3R) macrophages with anti-*I-J*^{k} serum did not significantly inhibit the response. Similarly, if B10.A(5R) macrophages were blocked with anti-*I-J*^{k} serum and then cultured with F_1 T-B cells, the PFC response was significantly inhibited. Treatment of the B10.A(5R) macrophages with anti-*I-J*^{b} serum had no effect.

This experiment supported the previous experiments that indicated the requirement for Lyt-1^+,2^-,3^- T_H recognition of an *I-J*-subregion product expressed on antigen-presenting macrophages. Thus, the blocking of the interaction between macrophage *I-J* product and T cell receptor by anti-*I-J* antibodies occurs at the level of the macrophage. Furthermore, the results of these experiments are interpreted as indicating restriction at the level of the T cell for specific receptors able to recognize macrophage membrane structures of *I-J*-subregion origin.

Discussion

It is well documented that there are genetic restrictions operative in the interaction between antigen-presenting macrophages and T cells (1–15)[1] and it is clear that these restrictions reside in the *I* region of the *H-2* gene complex (1–3, 6–8, 12–14, 24–28).[1] Opinions differ, however, as to the exact *I* subregion(s) responsible for the macrophage recognition structure. In part, the differences in findings may be ascribed to the use of different experimental models, different immunogens, and/or the level of primary or secondary response.

In previous publications from this laboratory, experiments were described that documented that the required macrophage in an in vitro antibody response was Ia$^+$, which expressed determinants of all known *I* subregions (13, 16, 17). Furthermore, only antibodies raised against determinants of the *I-J* subregion could effectively block macrophage recognition by Lyt-1^+,2^-,3^- T cells (13, 14, 16).[1] Anti-Ia antibodies

reactive with determinants of *I-A*, *I-E*, or *I-C* subregions always required complement and, therefore, deletion of the required macrophage subpopulation to inhibit the primary in vitro antibody response to BRBC.

The experiment reported in this manuscript, confirm the specific nature of the anti-*I-J* serum blocking of the macrophage-T cell interaction. Blocking of the response was only observed when the anti-*I-J* antibodies used to pretreat the F_1 macrophage were specific for the phenotype of the Lyt-1$^+$,2$^-$,3$^-$ T cells used to reconstitute the response. Simple antibody pretreatment of the macrophages did not alter their function because they were perfectly capable of interacting wth F_1 T cells or T cells of the parental phenotype that was not blocked by the anti-*I-J* antibodies. Thus, the experiments with anti-*I-J* serum-blocked F_1 macrophages are interpreted as demonstrating genetic restriction at the T cell level for syngeneic *I-J*-region determinant(s).

The observation that anti-*I-J* serum-treated F_1 macrophages function normally when cultured with F_1 T-B cells and with parental T-B cells syngeneic for the unblocked *I-J* phenotype was further evidence that the lack of response in cultures with anti-*I-J* serum-pretreated macrophages was not the result of specific or nonspecific suppression. The F_1 experiments were consistent with previous experiments in which cells harvested from culture dishes that contained anti-*I-J* serum-blocked macrophages were cocultured with normal spleen cells and antigen. These cocultured cells failed to suppress the response of normal cells.[1] Taken together, these various experiments support the argument that the blocked response was the result of anti-*I-J*-antibody effect on macrophages presumably by interfering with the ability of the T cell receptor to recognize an *I-J*-subregion product.

As in previous experiments, the effect of anti-*I-J* serum was demonstrated with only a brief incubation of antibody and macrophages after which the macrophages were blocked with mitomycin-C and washed repeatedly in fresh medium. Although a similar effect was demonstrated with the IgG fraction of the serum present during the entire culture period (J. E. Niederhuber and P. Allen. Unpublished data.), this was not necessary and suggested that the effects of anti-*I-J* antibodies were on an early event that involved antigen presentation and T cell recognition of the *I-J*-subregion determinant.

The results of the experiments presented here and in previous publications quite clearly implicate a product of the *I-J* subregion as the macrophage recognition unit for the T_H cell. These results differ from the reports of other investigators (24, 26–28) who have found a requirement for *I-A*-subregion identity between the antigen-presenting macrophage and the T_H cell. The early studies on *H-2* restrictions for macrophage-T cell cooperation were subject to criticism because of potential allogeneic effects arising from the mixing of histoincompatible cells. Recently, experiments with bone marrow chimeric mice to eliminate negative allogenic effects have also demonstrated a requirement for *I-A*-subregion identity between macrophages and T cells involved in antibody responses to hapten-carrier conjugates (24, 26). We have previously noted that in the in vitro antibody response to BRBC, mixing macrophages, and T-B cells differing at the *I-A* subregion resulted in an enhanced PFC response, whereas no PFC response was observed when macrophages and T-B cells differed only at the *I-J* subregion (13).

It is likely that differences in the requirements for *I*-region restrictions at the level of macrophage-T cell interaction differ depending upon the conditions of the response.

For example, the nature of the immunogen (multideterminant or structurally defined copolymers; particulate or soluble) and the level of response (primary or secondary) are important to the requirements for macrophage-T cell interaction. Furthermore, the experimental model (antigen-induced T cell proliferation, delayed-type hypersensitivity, or antibody synthesis) and the method of antigen presentation (macrophage bound) will also determine the restrictions observed at the level of macrophage-T cell cooperation.

The approach taken in the experiments presented here and in previous publications has been to interfere with the macrophage-T cell interaction by blocking Ia determinants with specific anti-Ia sera (13, 14, 17, 18, 29).[1] It is important to note that antisera to the stimulating antigen when used to pretreat the T cell population or the macrophage fraction has not altered the response (30, 31) (J. E. Niederhuber. Unpublished results.). Only antisera directed at Ia determinants has had a blocking effect and, in fact, only at the level of the macrophage. Our original report on the effect of anti-Ia sera on in vitro antibody responses involved the pretreatment of whole spleen cells (29). This effectively blocked both primary and secondary in vitro responses to erythrocyte antigens.

Anti-Ia sera were also found to block the in vitro stimulation of primed T cells by antigen-pulsed macrophages as measured by tritiated thymidine incorporation (28, 32). In the mouse, anti-Ia sera specific for *I-A* and *I-C* subregions blocked the tritiated thymidine incorporation of immune peritoneal exudate, T lymphocyte-enriched cells (PETLES) to stimulation with a terpolymer of L-glutamic acid, L-lysine, and L-phenylalanine (27). In these experiments, it was observed that anti-*I-A* serum also blocked the PETLES response to purified protein derivative (27). We have also observed that anti-*I-A* serum treatment of GAT-pulsed macrophages inhibits the ability of these cells to stimulate a proliferative response of GAT-immune T cells (J. E. Niederhuber. Manuscript in preparation.). Thus, our results and those of others cited here indicate a difference in the required macrophage Ia recognition unit dependent upon the antigen and in whether one uses the T cell proliferative assay or the in vitro antibody response.

It should also be noted that these observations support the hypothesis that macrophages present antigen fragments rather than intact antigen but do not determine the relationship between the Ia molecule(s) and the processed antigen fragments. Whether the T_H cell is stimulated by an Ia antigen fragment complex or by separate recognition of Ia determinants and antigen fragments will require further study.

Finally, it is important to state that the preparation of antibodies directed against gene products of the *I-J* subregion requires some special considerations not usually involved in mouse alloantisera production. Because the percentage of *I-J*$^+$ cells is very small in normal lymphoid cell populations, anti-*I-J* serum cannot be tested for activity by the standard microcytotoxicity assay. We have closely monitored the activity of anti-*I-J* reagents by testing their ability to block macrophage function in our in vitro antibody assay system and have determined that it is much more difficult to produce an active batch of anti-*I-J* serum than other *I* subregion-specific antisera. To produce an active anti-*I-J* serum such as B10.A(3R) anti-B10.A(5R), or B10.A(9R) anti-B10.HTT serum, it has been necessary to increase the donor:recipient ratio of immunizing cells and to immunize a minimum of 8–10 wk before bleeding the recipient mice. Anti-*I-J* sera produced in this manner can be purified on a protein-A

Sepharose-4B column (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.), which indicates that the blocking antibody was of the IgG class, most likely the IgG_{2a} subclass. The difficulty in producing active anti-I-J serum should always be kept in mind when interpreting experiments involving anti-I-J reagents.

Summary

The effect of specific anti-I-J reagents on macrophage-T cell interactions was studied in an in vitro antibody response to burro erythrocytes. Macrophages were prepared from the spleens of F_1 hybrid mice whose parental strains differed at the I-J subregion. Two F_1 hybrids were used for these experiments, [B10.A(3R) × B10.A(5R)]F_1 and [B10.S(9R) × B10.HTT]F_1. F_1 macrophages responded equally well with F_1 T-B cells or with T-B cells of either parental strain. When F_1 macrophages were pretreated with anti-I-J serum (without complement) specific for one parental haplotype, they were only able to cooperate with T helper (T_H) cells of the unblocked haplotype and with F_1 T_H cells. Identical results were obtained with $(J^b × J^k)F_1$ and $(J^s × J^k)F_1$ mice. The results indicate that T_H cells possess genetically restricted receptors for macrophage I-J-subregion gene products and that the interaction between this receptor and the macrophage I-J-subregion determinant is essential for the initiation of a primary in vitro antibody response to an erythrocyte antigen.

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