Xenogeneic antiserum to soluble products from activated lymphoid cells inhibits interleukin 1-mediated functions in the helper pathway of cytolytic-effector-cell differentiation

(interleukin 1 receptor/interleukin 2/skin graft/helper factor)

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ABSTRACT We have produced an antiserum that inhibits interleukin 1-mediated functions in immune responses. Skin graft-induced helper factor-containing supernatant (SgHFS) was used as immunogen in rats. The resultant antiserum was immunosuppressive of T-cell functions both *in vivo* and *in vitro*. We have further studied the effects of this antiserum on cell surface molecules that are involved in the generation of cytolytic effector T cells. Rat anti-SgHFS inhibited the differentiation of precytolytic effector cells in mixed lymphocyte cultures by blocking the helper-cell pathway. Both the level and the kinetics of interleukin 2 production were affected as the duration of rat anti-SgHFS pretreatment was increased. Interleukin 1 production after 24 hr in culture was unaffected. Monokines, including partially purified interleukin 1, actively compete with the rat anti-SgHFS by activating helper cells and thus circumvent suppression. Rat anti-SgHFS inhibits interleukin-1-mediated functions by a time-dependent active process. Thus, the target cell surface molecule(s) affected by the rat anti-SgHFS are associated with interleukin 1 function and may be the interleukin 1 receptor.

The generation of cytolytic T lymphocytes (CTL) involves a series of distinct cellular and soluble protein interactions that can be readily divided into two pathways: (i) activation of the helper T-cell pathway and subsequent release of helper/differentiation factors and (ii) activation and differentiation of precursor cytolytic T cells. The helper T-cell pathway is activated by at least two signals: antigen and interleukin 1 (IL-1) (1-3). The cytolytic T-cell pathway precursors require antigen and helper signals in order to differentiate into cytolytic T cells (4, 5). The role of IL-1 in the precytolytic pathway has not been defined. In the antigen-primed helper T-cell pathways, IL-1 serves as a necessary signal to trigger the production of interleukin 2 (IL-2) (2) and for the expression of IL-2 receptors (29). IL-2, also called T-cell growth factor, supports the proliferative expansion of antigen-activated cells both in the helper T-cell pathway and in CTL differentiation (6, 7). Other lymphokine/monokine factors besides IL-1 and IL-2 are required for the generation of CTL (4, 5, 8-14). The role of IL-1 in the production of these other lymphokines/monokines has not been determined.

One approach used to study the pathway of CTL differentiation has been to generate antiserum or monoclonal antibodies against antigen-primed T cells or their secreted/shed products. These antibodies are then used to further define the cell surface structure(s) involved in cell-cell collaboration as well as the function of various soluble factors involved in the differentiation of CTL. The contribution of a number of cell surface markers in both human and murine

CTL activities as well as the lymphokine IL-2 have been studied using this approach (15-21).

We have described an antiserum that was raised in rats against skin graft-induced helper factor-containing supernatant (SgHFS) and have utilized this rat anti-SgHFS to examine the differentiation pathways of precytolytic effector T cells (11, 22). The immunogen, SgHFS, was obtained from cells in the lymph nodes of C57BL/10 mice draining the site of B10.A(18R) skin grafted 6 days previously. The SgHFS has been analyzed functionally and shown to contain antigen-specific helper factors involved in the generation of cytolytic effector cells to the H-2D^d antigens. More recently, it has been found that the SgHFS contains major histocompatibility complex-restricted factors that are active in the induction of IL-2 synthesis (23). SgHFS obtained 6 or 8 days after grafting contains no detectable IL-2.

The rat anti-SgHFS inhibits a variety of T-cell-mediated responses *in vitro* and suppresses graft rejection *in vivo* (22). In the study reported here, we examined the mechanism involved in the inhibition of CTL generation by the antiserum. Pretreatment of the responders (precytolytic and helper T cells) but not the stimulators (antigen source) resulted in complete inhibition of CTL generation. Control levels of IL-1 were detected in the supernatants of pretreated cultures. A marked effect was observed, however, on the kinetics of IL-2 production and quantity of IL-2 produced. Antiserum pretreatment of responder cells either following incubation of cells with IL-1-containing supernatants or in the presence of partially purified IL-1 resulted in recovery of their capacity to differentiate into effector cells. These data suggest that the antibodies inhibited CTL differentiation by binding either to the IL-1 receptor, or to a molecule closely associated with IL-1 function, or to a linked membrane molecule.

MATERIALS AND METHODS

Animals. Female mice of strains C57BL/10 SgSn/J (H-2^b), B10.D2 (H-2^d) and DBA/2 (H-2^d) were obtained from The Jackson Laboratory. B10.A(18R) (H-2ⁱ/18) mice were obtained from J. Stimpfling (The McLaughlin Research Institute, Great Falls, MT) and bred locally. Fisher F-344 rats were obtained from Harlan Sprague Dawley, Indianapolis, IN.

Reagents. Complete medium (CM) to generate CTL consisted of RPMI 1640, 10% heat-inactivated human serum (56°C, 30 min), 2.0 mM L-glutamine, and antibiotics. Partial-

Abbreviations: CTL, cytolytic T cell(s); IL-1 and IL-2, interleukins 1 and 2; SgHFS, skin graft-induced helper factor-containing supernatant; RCAS, supernatant from rat spleen cells stimulated with concanavalin A; CM, complete medium; FS6, supernatants from concanavalin A-stimulated T-hybridoma FS6-14.13; B10, C57BL/10.

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ly purified IL-1 was obtained from Genzyme, Boston, MA. Crude IL-1-containing supernatants were obtained from the cell line P388D₁ (American Type Culture Collection). IL-1 activity was quantitated in the thymocyte proliferation assay (24). A stimulation index of 2 has been arbitrarily given the value of 1 unit of IL-1. The FS6-14.13 T-cell hybridoma (P. Marrack and J. Kappler, National Jewish Hospital, Denver, CO), the mixed lymphocyte reaction, and concanavalin A-stimulated rat spleen cells were utilized to prepare lymphokine-enriched supernatants. Concanavalin A activity was blocked by the addition of 0.3 M methyl α -D-mannopyranoside. CTLL/20.E1 IL-2-dependent cells (K. Smith, Dartmouth College, NH) were used to quantitate IL-2 levels (25). One unit of IL-2 was arbitrarily defined as the amount of a standard RCAS (supernatant from concanavalin A-stimulated rat spleen cells) that gave 50% maximum stimulation.

Rat Anti-SgHFS. SgHFS was obtained from C57BL/10 recipients of B10.A(18R) skin grafts 6 days after engraftment (11). SgHFS was emulsified in complete Freund's adjuvant and injected into Fisher F-344 female rats (6–7 weeks old). After 3 weeks, the rats were given weekly booster injections; they were bled after a minimum of six immunizations and the antiserum was pooled.

CTL. Mixed lymphocyte reaction cultures were incubated at a responder to stimulator ratio of 2:1. After 5 days, contents of the culture wells were pooled, and viable cells were counted and then titrated against a constant number of ⁵¹Cr-labeled target cells as described (11).

Pretreatment of Responder Cells or Stimulator Cells with Antiserum. Either responder or stimulator cells (10^7) were collected by centrifugation and resuspended in 1.0 ml of RPMI 1640 medium containing 5% heat-inactivated human serum. In some experiments, various lymphokine/monokine supernatants were admixed. The rat anti-SgHFS or normal rat serum was added (0.01 ml/tube), and the tubes were incubated at 37°C in a 5% CO₂ atmosphere for various times. The cells then were collected, washed twice with RPMI 1640, mixed with the appropriate untreated cells, washed again, and incubated in complete medium.

RESULTS

Rat anti-SgHFS exhibits immunosuppressive effects that are associated with T-cell proliferative responses; namely, (i) inhibition of responses to the T-cell activators—concanavalin A, phytohemagglutinin, and allogeneic cells—but not to the

FIG. 1. Kinetics of anti-SgHFS-mediated suppression of CTL generation. C57BL/10 spleen cells were cultured with γ -irradiated B10.D2 cells for 5 days. CTL responses of cultures that remained untreated for 5 days are included (*). Normal rat serum (●) or rat anti-SgHFS (■) was added to a final concentration of 1% (A) or 0.5% (B) at time 0 or on day 1, 2, 3, or 4 of culture.

B-cell mitogen, bacterial lipopolysaccharide; (ii) prolongation of the mean survival time of H-2-disparate skin grafts; and (iii) inhibition of the differentiation and effector function of CTL. The antiserum does not inhibit the IL-2-dependent proliferation of CTLL/20.E1 cells. The rat anti-SgHFS lacks specificity for allogeneic determinants of either the donor or recipients of the original skin grafts (22).

Kinetics of Rat Anti-SgHFS-Mediated Suppression *In Vitro*. Complete inhibition of CTL generation was observed when excess rat anti-SgHFS was added on day 0 or day 1 of a 5-day mixed lymphocyte reaction culture (Fig. 1). Less inhibition was evident when rat anti-SgHFS was added at later times. Lower concentrations of antiserum inhibited CTL generation only when added at the initiation of cultures (Fig. 1). These studies demonstrated that the antiserum inhibited an early event in CTL activation.

Responder Cells Serve as Targets of Rat Anti-SgHFS-Mediated Inhibition. Responder or stimulator cells were pretreated with antiserum for 6 hr, then washed and cultured with appropriate cells. Pretreatment of C57BL/10 (B10) responders resulted in complete inhibition of CTL generation (Fig.

FIG. 2. Effect of rat anti-SgHFS pretreatment of responder cells versus stimulator cells on CTL generation. (A and C) Responder strain cells were pretreated for 6 hr with normal rat serum (●) or rat anti-SgHFS (■). (B and D) Stimulator strain cells were pretreated with rat anti-SgHFS either before (▲) or after (■) γ -irradiation or with normal rat serum (●). C57BL/10 (B10) cells served as responders to B10.D2 stimulators (A and B) or as stimulators to B10.D2 responders (C and D). CTL were quantitated at various effector to target cell ratios (11).

2A). Pretreatment of B10.D2 stimulators, either before or after γ -irradiation, did not decrease the amount of killing detected (Fig. 2B). The specificity of the antiserum was for the responders, as opposed to the strain of origin, since reversing the responder and stimulator strains resulted in complete inhibition of CTL generation only with the pretreatment of B10.D2 responders (Fig. 2C) and not B10 stimulators (Fig. 2D). These findings are also reproducible with other strain combinations (unpublished results).

Cultures then were set up in which aliquots of responder cells were pretreated with normal rat serum or rat anti-SgHFS for intervals ranging from 5 min to 4 hr. The inhibition of CTL generation was greater as the pretreatment time was increased (Fig. 3), suggesting therefore that the rat anti-SgHFS modulates a membrane-associated function required for CTL generation.

Effects of Rat Anti-SgHFS on Production of IL-1 and IL-2. Antigen-priming and IL-1 activation of the helper T-cell pathway are two of the earliest known events associated with active membrane processes. Antigen-priming of precytolytic T cells does occur in the presence of the rat anti-SgHFS, as shown by the generation of CTL after the addition of RCAS. We thus quantitated the levels of IL-1 and IL-2 produced to determine whether the rat anti-SgHFS had an effect on helper T-cell activation. At various times after the responder cells had been pretreated with rat anti-SgHFS, washed, and cultured with γ -irradiated stimulator cells, the contents of the culture wells were pooled, the cells were removed, and the supernatants were assayed for IL-1 and IL-2 activities. A progressive decrease in cytolytic activity was observed again with increasing duration of antiserum pretreatment (Table 1). No significant differences in IL-1 levels were detected in supernatants collected 24 hr after the initiation of culture. No IL-2 activity was detectable at 24 hr in assays with the IL-2-dependent cell line; therefore, we concluded that the thymocyte proliferation assay on 24-hr supernatant indeed was measuring an effect of IL-1. At later time points, however, IL-2 was detected, so that IL-1 levels at these times could not be accurately measured in the thymocyte proliferation assay. A marked effect on IL-2 production

FIG. 3. Kinetics of inhibition induced by rat anti-SgHFS pretreatment of responder cells. B10 spleen cells were pretreated for various times with normal rat serum (●) or rat anti-SgHFS (■) at a final concentration of 1% before incubation for 5 days with γ -irradiated B10.D2 cells.

Table 1. Quantitation of IL-1 and IL-2 levels in mixed lymphocyte reaction cultures with rat anti-SgHFS-pretreated responder cells

Pretreatment*	Time	% specific ^{51}Cr release†	IL-1,‡ units/ml	IL-2,§ units/ml				
				24	48	72	96	120
CM	4 hr	74.0	4.8	<0.1	4.1	2.4	1.4	1.5
NRS	4 hr	71.9	8.0	<0.1	4.1	2.0	1.5	2.9
Anti-SgHFS	5 min	64.7	4.4	<0.1	5.8	3.8	1.6	2.4
	20 min	32.2	5.2	<0.1	1.7	3.1	2.7	2.5
	40 min	24.6	7.2	<0.1	1.2	2.7	1.6	0.4
	1 hr	10.2	6.8	<0.1	1.4	1.1	0.8	1.0
	2 hr	12.0	4.8	<0.1	0.5	1.7	1.8	1.1
	4 hr	6.9	6.4	<0.1	<0.1	<0.1	0.9	0.1

*B10 responder spleen cells were washed after pretreatment and cultured with γ -irradiated B10.D2 cells. NRS, normal rat serum.

†Cytolytic activity detected after 120 hr in culture. Effector to target cell ratio was 20:1.

‡Thymocyte proliferation assay was used to quantitate IL-1 levels at 24 hr, when IL-2 levels were undetectable.

§CTLL/20.E1 IL-2-dependent proliferation assay was used.

was evident, with both a decrease in the quantity and a shift in kinetics. The maximum quantity of IL-2 detected in either of the control groups was 4.1 units/ml at 48 hr. Supernatants from responder cells pretreated for 2 and 4 hr contained peak IL-2 levels of 1.8 and 0.9 units/ml, respectively, at 96 hr. Pretreatment of responder cells, thus, did not affect IL-1 production but substantially affected IL-2 production. On the basis of these data, we postulated that the rat anti-SgHFS blocked the function of IL-1 by binding either to the IL-1 receptor or to a closely associated molecule on the IL-2-producing cells. If the rat anti-SgHFS blocks IL-1-mediated activation of helper T cells, then the exogenous replacement of various helper lymphokines would result in CTL generation.

Effect of Helper Lymphokines. Responder cells were preincubated with antiserum for either 1 or 4 hr, washed, and cultured with stimulator cells in medium supplemented with either 5% RCAS; 10% FS6-14.13 T-hybridoma supernatant; or 1, 10, or 20% P388D₁ supernatants. CTL activity was gener-

FIG. 4. Effect of lymphokines and monokines on responder cells pretreated with rat anti-SgHFS for 1 or 4 hr. B10 spleen cells were pretreated with normal rat serum (dashed lines) or rat anti-SgHFS (solid lines) for 4 hr (A) or 1 hr (B) and then mixed with γ -irradiated B10.D2 cells in CM containing the indicated supernatants.

ated in cultures supplemented with RCAS and T-hybridoma supernatant (Fig. 4 A and B). A small but significant amount of cytolytic activity was detected when 1% or 10% P388D₁ supernatant was added to cultures with responder cells that had been pretreated for 1 hr. The addition of 20% P388D₁ had little effect on CTL generation. Responder cells pretreated for 4 hr were unresponsive to the addition of any concentration of P388D₁ supernatant. Nonspecific helper factors and mixed lymphocyte reaction supernatant were also able to induce the differentiation of CTL (data not shown). Lymphokines that function after the IL-1 signal in the helper pathway, therefore, were able to override the inhibitory effects of rat anti-SgHFS. The data obtained with IL-1-containing P388D₁ supernatants further suggest that the rat anti-SgHFS actively induces a time-dependent resistance to the IL-1- or macrophage factor-mediated signals. These data correspond with the kinetic experiments presented in Fig. 3, which also indicated that the rat anti-SgHFS-mediated inhibition involves active metabolic processes.

Effect of Monokines and Lymphokines During Pretreatment with Rat Anti-SgHFS. Various concentrations of monokine-(P388D₁) and lymphokine-containing supernatants were mixed with rat anti-SgHFS during the pretreatment of responder cells. Only the monokine supernatants effectively blocked the rat anti-SgHFS pretreatment. Inhibition of CTL generation in these cultures was substantially less than that observed in cultures with cells pretreated only with the rat anti-SgHFS (Fig. 5A) or in the presence of lymphokine supernatants (Fig. 5B). We have shown, in other experiments, that 1% P388D₁ supernatant (2 units of IL-1 activity) was the minimum quantity that would reverse the inhibition of CTL differentiation (data not shown).

Rat Anti-SgHFS Pretreatment of Responder Cells in a Two-Step Fashion. Although the rat anti-SgHFS exhibited little reactivity against P388D₁ supernatant in an ELISA assay (22), we considered the possibility that molecules in the supernatant were binding the antibodies and thereby inhibiting their interaction with cell surface determinants. Responder cells, thus, were incubated with either P388D₁ supernatant

FIG. 5. Effect of lymphokines and monokines during rat anti-SgHFS pretreatment of responder cells. (A) B10 responder cells were pretreated for 2 hr with 1% normal rat serum (●), 1% rat anti-SgHFS (□), or a mixture of 1% rat anti-SgHFS with P388D₁ monokine-containing supernatant at 10% (▲), 5% (▼), or 1% (○). (B) As in A, except that the mixtures had FS6-4.13 lymphokine-containing supernatant at 10% (▲), RCAS at 5% (▼), or SgHFS at 20% (■). After pretreatment (A and B), cells were washed and cultured with γ -irradiated B10.D2 cells for 5 days.

FIG. 6. Effect of monokine pretreatment of responders prior to rat anti-SgHFS (A) or in conjunction with rat anti-SgHFS (B). (A) B10 responder cells were pretreated for 2 hr with monokines [P388D₁ supernatant at 10% (▲)] or lymphokines [FS6-14.13 supernatant at 10% (○)] or CM (●, ■). The cells then were washed and further pretreated for 2 hr with normal rat serum (●) or rat anti-SgHFS (▲, ○, ■) before being incubated for 5 days with γ -irradiated B10.D2 cells. (B) As in A, except that the reagents were mixed together and the cells pretreated for a single 2-hr period before incubation with B10.D2 cells.

or FS6-14.13 lymphokine supernatant before pretreatment with the rat anti-SgHFS. CTL were generated only in cultures that had been incubated with P388D₁ supernatant before pretreatment with rat anti-SgHFS (Fig. 6A). Preincubation of responder cells with lymphokines had no effect. Results similar to these were obtained when either rat anti-SgHFS and P388D₁ supernatant or rat anti-SgHFS and FS6-14.13 supernatant were mixed together before pretreatment (Fig. 6B). It can be concluded, thus, that incubation of responder cells with monokines (P388D₁ supernatants), either before or during pretreatment with rat anti-SgHFS, blocked

FIG. 7. Effect of purified human IL-1 (pIL-1) on rat anti-SgHFS pretreatment of responder cells. B10 responder cells were pretreated for 2 hr with CM (○) or with rat anti-SgHFS at 1%, either alone (■) or in mixtures with purified human IL-1 (Genzyme) at 10% (*), control diluent (Genzyme) at 10% (○), or P388D₁ supernatant at 10% (▲). Cells were washed before culture in CM with γ -irradiated B10.D2 cells. Data from two experiments are presented (A and B).

the inhibitory effect, whereas preincubation with lymphokine-containing supernatants did not.

Effect of Purified IL-1 on Rat Anti-SgHFS Pretreatment. The crude P388D₁ supernatants may contain a number of monokines that affect membrane-associated processes. Therefore, we preincubated the responder cells in the presence of purified human IL-1. In two separate experiments, purified human IL-1 was able to overcome the inhibitory effect of rat anti-SgHFS (Fig. 7). CTL activity obtained in cultures pretreated with rat anti-SgHFS and purified IL-1 was similar to the levels observed in cultures pretreated with antiserum and constitutively produced IL-1 from the P388D₁ cell line.

DISCUSSION

Cell surface molecules that function in IL-1-mediated signals on activated helper T cells are shed during antigen-priming. Xenogeneic antisera raised against such shed or secreted products contain antibodies that block IL-1 function. These products are in cell-free supernatants obtained from skin graft-primed mice. Several observations suggest that the rat anti-SgHFS blocks receptor-associated functions. First, pretreatment of responding cells with monokine-containing (P388D₁) supernatants reduces the inhibition by the antiserum. Second, the rat anti-SgHFS must interact with responding cells for at least 2 hr in order to accomplish complete inhibition. Third, the addition of monokine-containing supernatants or purified IL-1 before that time has elapsed results in a substantial decrease in the inhibitory effect of the rat anti-SgHFS. Apparently, IL-1 actively competes with the antiserum by actuating the target molecules on the cell surface. Furthermore, antibody-binding is not the only requirement for inhibition to occur. Rather, the responding cell population becomes desensitized to the IL-1 signal with increasing time of treatment with antiserum. Other biological systems that involve receptor-ligand interactions exhibit similar kinetics. Antiserum directed against the insulin receptor of an adipocyte-like cell line, for example, results in a decrease in responsiveness to insulin, as measured by 2-deoxyglucose transport, with increasing periods of pretreatment with the anti-receptor serum. Further studies demonstrated that although these cells had become unresponsive to insulin, they remained responsive to other stimulants such as spermine and vitamin K₃ (4-amino-2-methyl-1-naphthol) (26–28). Conclusive evidence that we indeed have been able to generate antibodies directed against the IL-1 receptor must await either the availability of radiolabeled purified IL-1 or the production of a monoclonal antibody. It is clear, however, that the rat anti-SgHFS recognizes molecules associated with IL-1 functions in CTL responses. We should point out too that the antiserum has immunosuppressive effects *in vivo* and prolongs median skin graft survival times (22).

The data presented herein also suggest that the rat anti-SgHFS directly inhibits the helper pathway as opposed to the precytolytic-T-cell differentiation pathway. The addition of several nonspecific helper factor- and IL-2-containing supernatants to rat anti-SgHFS-pretreated responders resulted in the generation of cytolytic effectors. The precursor cells, therefore, had become activated by antigen despite the pretreatment with antiserum.

An effect of the rat anti-SgHFS on the helper pathway was further evidenced by the substantial decrease in the quantity of IL-2 detected. The fact that the IL-1 levels produced after 24 hr in culture were equal to control levels indicated that the activation of macrophages to produce this monokine was unaffected. We conclude that IL-1-induced synthesis of IL-2 was blocked.

The addition of exogenous lymphokines circumvented the inhibition of IL-1-mediated function, but addition of macrophage-cell line products could not. In further experiments (data not included) we added irradiated antigen-primed cells to rat anti-SgHFS-pretreated responders and were able to recover functional cytolytic effectors. These data again support the conclusion that the rat anti-SgHFS directly inhibited the helper pathway and not the pre-effector cell pathway. The inhibition of CTL generation by the rat anti-SgHFS, therefore, resulted from a lack of adequate helper signals. Our data clearly support other published reports of a requirement for IL-1 in helper-cell activation and, moreover, suggest that IL-1 is not required as a direct signal in the precytolytic-T-cell differentiation pathway.

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