MECHANISMS OF REGULATION OF CELL-MEDIATED IMMUNITY

III. The Characterization of Azobenzenearsonate-Specific Suppressor T-Cell-Derived-Suppressor Factors*

By MARK IRWIN GREENE, BRUCE ALLEN BACH,‡ AND BARUJ BENACERRAF

From the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

Suppressor T cells (STC)[1] play decisive roles in the regulation of the immune response to many antigens (1–4). Suppressor T cells have been shown to elaborate antigen-specific factor(s) (2, 5–7) which have been subjected to immunochemical analysis and found to share many general properties. The suppressor factors (SF) studied in the greatest detail are proteins (7) with mol wt of 33–68,000 and, in many cases, bear determinants encoded by the major histocompatibility complex (5, 6, 7–9). The nature of the antigen-recognition site remains unclear. When studied, such SF do not appear to bear conventional determinants of immunoglobulin (Ig) light or heavy chain, because polyvalent antisera against normal Ig (5–8) or the $F(ab')_2$ fragment do not bind the SF. However, antisera to V_H-region determinants have not been thoroughly assessed for binding activity to regulatory molecules.

Recent studies from our laboratory have analyzed the precise requirements for the induction and suppression of *p*-azobenzenearsonate (ABA)-specific delayed type hypersensitivity (DTH) in A/J mice (10). ABA was directly coupled to syngeneic spleen cells and these used as immunogens. These studies revealed that major histocompatibility complex (MHC) gene products played a critical role in the specificity of DTH-induced by ABA-cells. As has been shown previously by Miller and Claman (11) as well as by ourselves (12), the intravenous administration of hapten-coupled syngeneic cells stimulates the emergence of hapten-specific suppressor T cells (10, 12). Furthermore, the ABA-specific suppressor T cells produce an ABA-specific SF which suppresses ABA-specific DTH. The availability of ABA-specific suppressor T cells and TsF is particularly interesting in light of the observation that a major cross-reactive idiotype (CRI) has been found on 20–70% of anti-ABA antibodies produced by A/J mice after immunization with ABA-keyhole limpet hemocyanin (KLH) (13). In this study, we will describe the production and immunochemical properties of ABA-specific suppressor T-cell-derived SF, including the expression on ABA SF of determinants controlled by the MHC genes on the XVIIth chromosome. In a companion paper we will investigate the presence of ABA cross-reactive idiotype on ABA-specific

---

* Supported by grant P01 CA-14723 from the Department of Health, Education, and Welfare.

‡ Supported by a National Research Service Award from the U.S. Public Health Service, CA-0194-03.

[1] *Abbreviations used in this paper:* ABA, *p*-azobenzenearsonate; BBS, borate-buffered saline; CRI, cross-reactive idiotype; DTH, delayed type hypersensitivity; FCS, fetal calf serum; FGG, fowl gamma globulin; KLH, keyhole limpet hemocyanin; MEM, minimum essential medium; MHC, major histocompatibility complex; Rb, rabbit; SF, suppressor factor; STC, suppressor T cells.

TsF (14). Rabbit antisera directed against idiotypic determinants will be shown to be of importance in the definition of similarities between ABA-specific Ig, and ABA-specific T-cell-derived suppressor products.

Materials and Methods

Mice. A/J (H-2^a), BALB/c (H-2^d), C57BL/6 (H-2^b), B10.A (H-2^a), and C.AL-20 female mice 8-10 wk old were used in these experiments. The C.AL-20 mice, originally bred by Michael Potter, were provided from stocks maintained by Dr. A. Nisonoff at Brandeis University. All other mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Antigen and Cell-Coupling Conditions. These have been described in detail elsewhere (10). Briefly, a 40-mM solution of ABA diazonium salt was prepared from arsanilic acid (Eastman Kodak Co., Rochester, N. Y.). The ABA solution was activated as previously described and conjugated to single-cell suspensions of erythrocyte-free splenocytes at a final concentration of 10 mM ABA. After washing in fetal calf serum (FCS)-free medium, the ABA-coupled cells were used to induce DTH as previously described (and outlined in Fig. 1) or alternatively used to induce suppressor T cells by intravenous injection (10).

Priming for DTH. Briefly, to induce DTH to ABA, 3×10^7 ABA-coupled syngeneic cells were injected subcutaneously into separate sites on the dorsal flanks of mice. Challenge was performed 5 d later by injecting 25 μl of a solution containing 2×10^7 ABA-coupled syngeneic cells or 25 μl of 10 mM ABA diazonium salt into the left footpad.

Preparation of Antisera

Preparation of alloantisera. The preparation of B10 anti-B10.A (anti-K^kI-A^kI-B^kI-$J^kIC^dS^dD^d$) and B10.D2 anti-B10.A (anti-K^kI-A^kI-B^kI-J^kI-E^k) was described previously (7, 8). Briefly, sera were obtained after 6-10 biweekly ip. injections of $2-5 \times 10^7$ spleen cells. The cytotoxic titer of antibodies from these sera was determined by the ^{51}Cr release assay as described previously (7) using B10.A spleen cells as targets. For 50% lysis of 10^5 appropriate target cells with 0.1 ml of antiserum, the cytotoxic titer were B10.D2 anti-B10.A 1:1280 and B10 anti-B10.A > 1:1280.

A/J anti-ABA antibodies. A/J anti-ABA antibodies were generated by immunizing mice with ABA-KLH as described by Nisonoff et al. (13).

Preparation of rabbit anti-mouse F(ab')$_2$ serum. Antiserum to mouse F(ab')$_2$ was produced in rabbits by three intramuscular biweekly injections of 680 μg of F(ab')$_2$ fragments of mouse IgG$_2$ in Freund's complete adjuvant (FCA). For this purpose F(ab')$_2$ fragments were prepared in the following manner. 50 ml of pooled whole mouse serum were treated with ammonium sulfate at 50% saturation and the precipitate was redissolved in borate-buffered saline (BBS); this process was repeated three times. The globulins so precipitated were dissolved in 5 ml of saline and dialyzed against BBS in the cold and the IgG$_2$ fraction was isolated by preparative agar block electrophoresis in barbital buffer (pH 8.6, μm-0.1), as described in a previous study (7). The F(ab')$_2$ fragments were then prepared by the method of Nisonoff (15) with slight modifications. Briefly, 20 mg of IgG$_2$ were incubated with 0.6 mg of pepsin (Sigma Chemical Co., St. Louis, Mo.) for 16 h at 37°C in acetate HCl buffer, pH 4.0, and the enzymatic digestion was stopped by adjusting the pH to 8.0 with 1 N NaOH; the reaction mixture was then dialyzed against BBS. The F(ab')$_2$ fragments were then freed of any undigested globulins by gel filtration through a Sepharose G-200 column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.). Further characterization by Ouchterlony diffusion employing appropriate antiserums, failed to detect the presence of Fc determinants.

Preparation of immunoadsorbents. Solid-phase immunoadsorbents were prepared according to the procedure of Cuatrecasas (17) with some modifications. In the case of rabbit anti-mouse F(ab')$_2$ antisera, the 7S globulins were isolated from whole serum by gel filtration on a Sephadex G-200 column before being coupled to activated Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.). ABA was coupled to fowl gamma globulin (FGG) according to the method described by Ju et al. (18). ABA-FGG, FGG, and a control serum consisting of normal rabbit serum were coupled to activated Sepharose 4B at a final concentration of 2 mg protein/ml of Sepharose 4B.

Anti-Thy 1.2 treatment. AKR anti-C3H (anti-Thy 1.2) serum was prepared according to

the method of Reif and Allen (16) and was used as described previously (10). Guinea pig serum was used as a source of complement and was absorbed extensively with murine splenocytes. At the optimal dilution of C there was no discernible cytotoxicity for A/J thymus cells; the cells were lysed by a specific B.10 anti-B10.A antiserum and C.

PREPARATION OF SUPPRESSOR FACTOR. A/J female mice 10 wk old were given an i.v. injection of 5×10^7 syngeneic spleen cells which had been coupled with azobenzenearsonate diazonium salt. 7 d after administration of ABA cells, the mice were killed and spleen and thymus cells were teased separately into single-cell suspensions and washed with Eagle's minimal essential medium (MEM), pH 7.2, which had been supplemented with 2 mM glutamine. Suppressor factor was produced from such cells according to one of three protocols.

PRODUCTION BY CELL DISRUPTION. (*a*) As has been previously described for other antigen-specific factors (19) suppressor cells were sonicated in a sonifer cell disrupter model W-140 E (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) using a standard microtip. 1×10^9 cells in a 2-ml volume were sonicated for 5 min at 50 W. The sonicates obtained were centrifuged at 400 *g* for 20 min and then immediately recentrifuged at 10,000 *g* for 1 h at 4°C in a Beckman model J centrifuge (Beckman Instruments, Inc., Fullerton, Calif.). After centrifugation such materials were adjusted to 5×10^8 cell eq per milliliter and frozen at −40°C. (*b*) Production by snap freezing and thawing was performed precisely as described by Greene et al. (7). Briefly, 2–4×10^9 washed splenocytes or thymocytes in a 2 ml of Hank's solution were subjected to alternate snap freezing at −78°C and thawing at 37°C; this was repeated four times and was followed by ultracentrifugation at 10,000 *g* for 1 h. In some cases, to eliminate products of low molecular weight, the soluble extracts were dialyzed against 1 liter of BBS.

PRODUCTION OF SUPPRESSOR FACTORS IN SUPERNATES OF SUPPRESSOR T CELLS. The method was that described by Zembala and Asherson (20) with some modifications. 6 d after the i.v. administration of ABA-coupled cells, the mice were challenged in all footpads with 25 μl of 10 mM ABA diazonium salt. 24 h later, the spleens and thymuses were removed under sterile conditions and single cell suspensions made by teasing the separate organs. Cells were resuspended at a concentration of 10^7 cells/ml in Eagle's MEM supplemented with 10% FCS, penicillin, streptomycin, and 10 mM glutamine. The cells were placed in Falcon tissue culture flasks (Falcon Labware, Div. of Becton Dickinson and Company, Oxnard, Calif.) and maintained at 37°C for 48 h in vitro. Supernates were gathered and concentrated by negative pressure dialysis in BBS, pH 8.0. The supernates were adjusted to volume equivalents derived from 5×10^8 cells/ml and frozen until used at −40°C.

MEASUREMENT OF FOOTPAD SWELLING. 24 h after the footpad challenge, DTH reactivity was assessed by measuring the footpad swelling using a Fowler micrometer (Schlesinger's, Brooklyn, N. Y.). The magnitude of the DTH reaction was expressed as the increment of thickness of the challenged left footpad as compared with the untreated right footpad. Responses are given in units of 10^{-3} in ±SEM from groups of five mice (Fig. 1). Statistical analysis of the difference of data obtained utilized the two-tailed Student's *t* test.

Results

Generation and Transfer of Suppressor Cells

SUPPRESSOR CELLS ARE T-CELL DERIVED. The ability to induce suppressor cells by the intravenous administration of ABA-coupled cells was previously documented (10). To define the phenotype of the suppressor cells capable of inhibiting the DTH response to ABA-coupled cells, we administered 5×10^7 ABA-coupled cells i.v. to A/J ($H\text{-}2^a$) mice. The spleen and thymuses were removed 7 d later and adoptively transferred to A/J mice at the time of subcutaneous immunization with ABA-coupled syngeneic cells. The suppressor cells virtually abolished the DTH response evoked by footpad challenge with ABA or ABA-coupled cells (10). Furthermore, as seen in Table I, the suppressor cells are thymus-derived as deduced by their sensitivity to anti-Thy 1.2 alloantisera and complement (C′) treatment in vitro, before adoptive transfer. Thus STC capable of limiting the DTH response to ABA cells are demonstrable in the thymus and spleen of A/J mice given ABA A/J cells i.v.

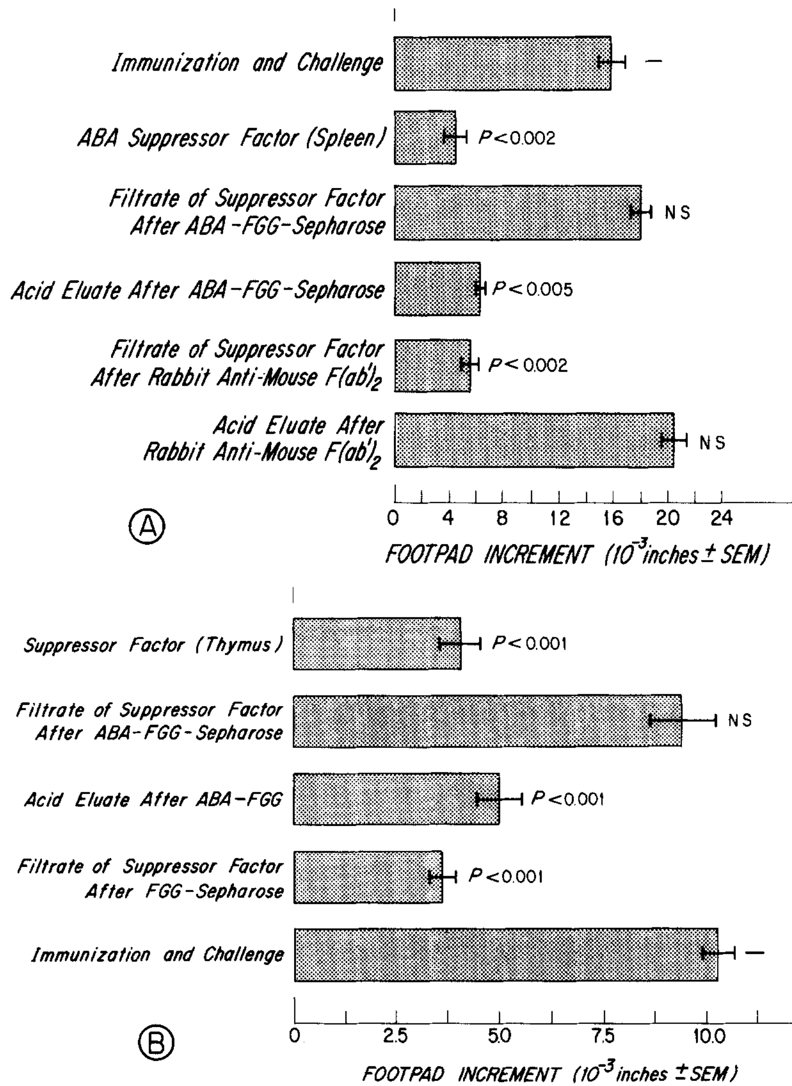

FIG. 1. Antigen-binding specificity of ABA factor(s). A/J mice were immunized subcutaneously with 3×10^7 ABA-derivatized syngeneic cells. Such mice were then given suppressor factor which had been fractionated as indicated. Suppressor factor alone at a dose of 2×10^7 cell eq/mouse, suppressor factor which had been absorbed to a Sepharose ABA-fowl gamma globulin column, and the acid eluate of such columns was assayed for both spleen (1A) and thymus (1B) factor. In addition, the filtrate and eluate of spleen factor which had been passed over a rabbit anti-mouse $F(ab')_2$ was assayed (1B).

SUPPRESSOR T CELLS PRODUCE SUPPRESSOR FACTOR(S). To determine if such STC contain subcellular elements capable of inhibiting DTH to ABA, STC were subjected to mechanical disruption by freeze-thaw. STC extracts were administered to normal A/J mice beginning at the time of immunization with 3×10^7 ABA-A/J cells subcutaneously. Administration of 2×10^7 cell eq of factor, (snap freeze-thaw preparation) was performed each day for five successive days. These mice were challenged 5 d later with either ABA-derivatized syngeneic cells or the diazonium salt

TABLE I

θ-Positive Thymocytes and Splenocytes from A/J Mice Given ABA-A/J Cells I.V. Suppress ABA-Specific DTH

Group	Immunization (subcutaneous)	Suppressor cells (intravenous)	Mean footpad increment* 10^{-3} in ± SEM	*P*
I	3×10^7 ABA-A/J	—	10 ± 0.5	—
II	3×10^7 ABA-A/J	5×10^7 Spleen suppressor cells	3 ± 0.7	<0.001
III	3×10^7 ABA-A/J	5×10^7 Thymus suppressor cells	4.6 ± 0.3	<0.001
IV	3×10^7 ABA-A/J	5×10^7 Spleen suppressor cells (NMS + C′)	3 ± 1.0	<0.001
V	3×10^7 ABA-A/J	5×10^7 Spleen suppressor cells ($\alpha\theta$ + C′)	8 ± 1.2	NS
VI	3×10^7 ABA-A/J	5×10^7 Normal A/J spleen cells	9.7 ± 1.2	NS
VII	—	—	1 ± 0.7	<0.001

* 25 μl of 10 mM ABA diazonium salt in the footpad.

of ABA in the footpad and the T-cell dependent reaction assessed 24 h later (10). As can be seen in Table II, either spleen or thymus STC-derived SF abolished the DTH response. Therefore, SF can be shown to exert profound inhibitory effects on the expression of ABA DTH. Normal thymic extracts, however, were found to be incapable of detectable suppression (data not shown).

SF PRODUCED BY A VARIETY OF TECHNIQUES ARE EQUALLY SUPPRESSIVE. To compare the biological activity of SF produced by a variety of techniques, A/J mice were injected intravenously with 5×10^7 ABA-A/J cells/mouse. One group of mice was challenged with ABA diazonium salt in the footpads and 24 h thereafter the spleen, thymus, and lymph nodes were removed under sterile conditions and placed in in vitro culture as described in Materials and Methods. STC supernates were concentrated and assessed for their ability to limit DTH. Alternatively, suppressor thymus or spleen cells were subjected to either sonication as previously described, or mechanical disruption accomplished by snap freezing and thawing. The sonicates or extracts were concentrated and extensively dialyzed and then assessed for inhibitory activity. As can be seen in Table III, the material produced in sonicates, extracts or supernates, when administered at doses of 2×10^7 cell eq/d per mouse for five consecutive days were equally capable of suppressing T-cell dependent DTH reactivity to ABA. Normal thymic or spleen extracts were found to have no discernible suppressive effects (data not shown).

ABA SF SPECIFICALLY INHIBITS ABA DTH. To determine the biological specificity of SF, the material was assessed for its ability to inhibit TNP- or ABA-specific DTH. Previously, we had demonstrated that ABA cell-induced STC were specific in their activity limiting only the ABA-DTH response and not the TNP-DTH response. A/J mice were primed with TNP-A/J or ABA A/J cells administered subcutaneously. Both groups of these mice received 2×10^7 C.E./d per mouse of ABA cell-induced SF on a daily basis and the DTH reactivity assessed 5 d later. As can be seen in Table IV, only ABA DTH was inhibited, whereas TNP-specific DTH was not affected. Thus, SF derived from STC induced by ABA conjugated cells specifically limits ABA DTH reactivity. The specificity of TNBS induced SF has been reported elsewhere (8, 20).

TABLE II

Suppressor Factors Are Produced by Suppressor Thymus and Spleen Cells

Exp	Group	Suppressor factor source	Immunization (subcutaneous)	Challenge (footpad)	Mean footpad increment 10^{-3} in ± SEM	P
I						
	I	—	3 × 10^7 ABA-A/J cells	ABA-A/J cells*	13.4 ± 1.8	—
	II	Thymus	3 × 10^7 ABA-A/J cells	ABA-A/J cells	4.5 ± 0.96	0.001
	III	Spleen	3 × 10^7 ABA-A/J cells	ABA-A/J cells	2.0 ± 1.4	0.001
	IV	—	—	ABA-A/J cells	3.0 ± 1.5	0.001
II						
	I	—	3 × 10^7 ABA-cells	ABA‡	10.33 ± 0.9	—
	II	Spleen	3 × 10^7 ABA-cells	ABA	3.1 ± 1.1	<0.005
	III	Thymus	3 × 10^7 ABA-cells	ABA	4.1 ± 1.0	<0.005
	IV	—	—	ABA	1.6 ± 1.3	<0.001

* 2 × 10^7 ABA cells in the footpad.

‡ 25 μl of 10 mM ABA diazonium salt in the footpad.

TABLE III

Suppressor Factors Are Found In Extracts, Sonicates, or Supernates of Suppressor T Cells

Group	Suppressor factor preparation*	Cell source of suppressor molecules	Immunization‡	Mean footpad increment 10^{-3} in ± SEM§	P
I	—	—	3 × 10^7 ABA A/J	14 ± 0.95	—
II	Sonicate	Thymus	3 × 10^7 ABA A/J	5.5 ± 0.9	0.005
III	Sonicate	Spleen	3 × 10^7 ABA A/J	4.4 ± 1.2	0.004
I	—	—	3 × 10^7 ABA A/J	17.5 ± 1.7	—
II	Supernate	Thymus	3 × 10^7 ABA A/J	4.75 ± 0.66	0.001
III	Supernate	Spleen	3 × 10^7 ABA A/J	5.5 ± 0.4	0.001
IV	—	—	—	3.2 ± 0.6	0.001
I	—	—	3 × 10^7 ABA A/J	13.4 ± 1.8	—
II	Freeze and thaw extract	Thymus	3 × 10^7 ABA A/J	4.5 ± 0.1	0.001
III	Freeze and thaw extract	Spleen	3 × 10^7 ABA A/J	2 ± 0.1	0.001
IV	—	—	—	3 ± 1.5	0.001

* 2 × 10^7 cell suppressor equivalents/day/mouse for 5 consecutive days.

‡ 3 × 10^7 hapten-derivatized cells s.c. day 0.

§ 2 × 10^7 hapten-derivatized cells in 25 μl day 5.

ABA CELL-INDUCED STC-DERIVED SF BIND SPECIFICALLY TO ABA. Having established the biological specificity of ABA cell-induced SF, we next examined the binding specificity of this SF. This was accomplished in two series of experiments. In the first, depicted in Fig. 1 a, b, SF were passed over immunoadsorbents prepared by coupling ABA-FGG to activated Sepharose 4B. The SF filtrate and the glycine-HCl eluate (pH 2.8) were collected, dialyzed against BBS (pH 7.0), and concentrated to original volumes before assay for suppressive activity. As can be seen, virtually complete elimination of the suppressive activity was accomplished by passage over ABA-FGG, which reflects the binding of SF to ABA. The eluates of these ABA-FGG columns were, on the other hand, completely suppressive for DTH to ABA. Control columns prepared with FGG coupled to Sepharose 4B were incapable of discernible interaction

TABLE IV

Suppressor-Cell-Derived-Suppressor Factors Induced by the Intravenous Administration of ABA Cells Inhibit Only ABA DTH

Group	ABA Suppressor* factor	Immunization‡	Challenge§	Mean footpad increment 10^{-3} in ± SEM	*P* value
I	--	TNP-cell	TNP-cell	16.2 ± 2.14	—
II	--	—	TNP-cell	4.5 ± 0.50	<.001
III	+	TNP-cell	TNP-cell	13.4 ± 1.15	<.288
IV	--	ABA-cell	ABA-cell	13.2 ± 0.92	—
V	--	—	ABA-cell	3.0 ± 1.53	<.001
VI	+	ABA-cell	ABA-cell	3.1 ± 1.42	<.001

* 2×10^7 cell suppressor equivalents/day per mouse for five consecutive days.

‡ 3×10^7 hapten-derivatized cells sub day 0.

§ 2×10^7 hapten-derivatized cells in 25 μl day 5.

TABLE V

ABA Cell-Induced Suppressor T-Cell-Derived-Suppressor Factors Can Be Absorbed on ABA-Derivatized Cells and Not on TNP-Derivatized Cells

Exp	Group	Suppressor* factor 5×10^8 cell equivalents	Absorption‡	Immunization‡	Footpad challenge	Mean footpad increment 10^{-3} in $\times 10^3$	*P*
I							
	I	—	—	3×10^7 ABA-A/J	ABA-A/J§	13.2 ± 0.9	
	II	Spleen	—	3×10^7 ABA-A/J	ABA-A/J	2.0 ± 1.4	<0.001
	III	Spleen	10^8 TNP cells	3×10^7 ABA-A/J	ABA-A/J	3.0 ± 0.41	<0.001
	IV	Spleen	10^8 ABA cells	3×10^7 ABA-A/J	ABA-A/J	9.5 ± 1.4	NS
	V	—	—	—	ABA-A/J	± .5	<0.001
II							
	I	—	—	3×10^7 ABA-A/J	ABA‖	12.5 ± 1.3	
	II	Spleen	—	3×10^7 ABA-A/J	ABA	4.9 ± 0.4	<0.001
	III	Spleen	10^8 TNP-cells	3×10^7 ABA-A/J	ABA	5.4 ± 0.2	<0.001
	IV	Spleen	10^8 ABA-cells	3×10^7 ABA-A/J	ABA	10.6 ± 1.3	NS
	V	—	—	—	ABA	2.9 ± 0.6	<0.001

* 5×10^8 suppressor cell equivalents were absorbed and fractionated on a volume basis to give 2×10^7 cell eq of absorbed suppressor cell molecules/mouse per day for 5 d.

‡ Suppressor molecules were absorbed with nonadherent hapten modified cells while being rotated for 1 h at 4°F.

§ 2×10^7 ABA/A/J cells in the footpad as a challenge.

‖ 25 μl of 10 mM ABA diazonium salt in the footpad as a challenge.

with SF. Hence, SF binds in a precise manner to the homologous antigen which was used for STC generation. Alternatively, SF were absorbed on TNP- or ABA-derivatized syngeneic nonadherent splenic lymphocytes for 1 h at 4°C. Thereafter, these SF were assayed for suppressive activity in the conventional manner. As is seen in Table V, SF absorbed on ABA cells were inefficient in limiting ABA-DTH, whereas SF absorbed by TNP-cell conjugates SF were quite inhibitory. These results similarly document the specificity of SF induced by ABA conjugated to syngeneic cells.

TABLE VI
Anti-ABA Antibodies Do Not Limit DTH

Group	Anti-ABA antibody*	Immunization	Challenge	Mean footpad response 10^{-3} in ± SEM	P
1	–	3×10^7 ABA A/J	ABA	10.2 ± 0.37	—
2	+	3×10^7 ABA A/J	ABA	9.4 ± 0.6	NS
3	–	—	ABA	2.5 ± 0.2	<0.001

* 5 μg of IgG anti ABA in 0.2-ml volume was administered to each of five A/J mice/daily for 5 consecutive d.

ABA-SPECIFIC SUPPRESSOR FACTORS DO NOT BEAR CONVENTIONAL IMMUNOGLOBULIN DETERMINANTS. The precise binding specificity of STC-derived SF to ABA suggested the possibility that SF might possibly represent immunoglobulin or fragments of immunoglobulin. To examine this possibility, reverse immunoadsorbents were prepared utilizing a rabbit (Rb) anti-mouse $F(ab')_2$ antiserum prepared by immunizing rabbits with pooled mouse $F(ab')_2$. Furthermore, the antiserum had been affinity-purified on a pooled mouse globulin immunoadsorbent and was shown to react with μ-, γ-, α-heavy chains and both κ and λ light chains (Mark I. Greene, unpublished observations). The SF was passed over two separate columns, one ABA-FGG, and the second, the above mentioned Rb anti-mouse $F(ab')_2$ immunoadsorbent. As can be seen in Fig. 1 a, the spleen SF was not removed by the anti $F(ab')_2$ column but was completely removed by the ABA-FGG. Control immunoadsorbent columns composed of normal rabbit sera which had been absorbed with A/J splenocytes, had no demonstrable binding activity to SF (data not shown). Thus, the antigen-specific T-cell derived SF does not bear conventional immunoglobulin markers.

ANTI-ABA ANTIBODIES ADMINISTERED INTRAVENOUSLY DO NOT LIMIT ABA DTH. Having established that a regulatory molecule derived from STC could specifically bind to ABA-FGG, we asked whether anti ABA antibody, with specificity analogous to SF, could limit ABA specific DTH. A/J mice were primed with 3×10^7 ABA cells subcutaneously; they simultaneously received i.v. 5 μg of pooled Ig containing anti-ABA antibody that had been obtained from A/J mice immunized with ABA-KLH. This injection was repeated daily for five consecutive days. As shown in Table VI, this did not discernibly affect the DTH response. It can be concluded that antibody with the same ABA specificity when given i.v. on a daily basis, cannot mimic the action of ABA-specific SF.

MOLECULAR SIZE OF ABA SF. To elucidate some of the properties of ABA SF, the size of the active moiety was determined by gel filtration through a precisely calibrated Sephacryl G-200 column. As can be seen in Fig. 2, the SF appears to be present in the fraction with mol wt 33-68,000, in agreement with previous results on the size of antigen-specific SF(3,8). It should be noted that some suppressive activity was also apparent in the smaller (<33,000) and the larger (>68,000) fractions.

ABA-SPECIFIC SF(S) CAN BE PRODUCED BY A VARIETY OF INBRED STRAINS. To investigate in a preliminary manner the ability of a variety of inbred strains to produce ABA-specific suppressor material, A/J(H-2^a) (Ig-1^e), C57B1/6 (H-2^b)(Ig-1^b), BALB/c(H-2^d)(Ig-1^a), CBA (H-2^k)(Ig-1^d), B10-A(H-2^d)(Ig-1^b) and C.AL-20(H-2^d)(Ig-1^d) were used. STC were generated in each strain by the use of ABA-derivatized syngeneic splenocytes administered intravenously. SF were then produced by mechanically

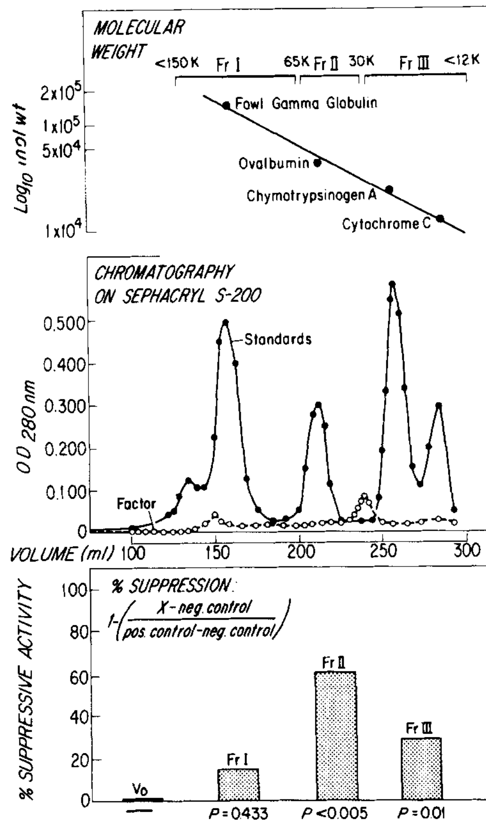

FIG. 2. 2×10^8 cell eq, of ABA factor, was fractionated over a Sephacryl S-200 column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway N. J.) using BBS buffer, pH 7.2. The column was calibrated with fowl gamma globulin, ovalbumin, chymotrypsinogen, and cytochrome C. To assay such fractions, the optical density was measured for each 5-ml fraction and material corresponding to molecular weight fractions, 150,000-65,000, 65,000-30,000, and 30,000-12,000 was pooled and vacuum concentrated. Such materials were injected into groups of five mice as shown in the protocol presented in Fig. 1. The percent suppression was calculated according to the formula:

$$\text{Percent suppression} = 1 - \left[\frac{\text{experimental} - \text{negative control}}{\text{positive control} - \text{negative control}}\right].$$

disrupting STC, and were tested in the strain of origin. In this limited study presented in Table VII, it would appear that any strain, regardless of H-2 or Ig-1 haplotype, is capable of generating SF which are effective in limiting DTH to ABA in syngeneic recipients.

ABA-SPECIFIC SF BEAR H-2 MHC-ENCODED DETERMINANTS. To analyze for the presence of determinants encoded by the H-2 MHC, A/J STC-derived SF were passed over a number of immunoadsorbents prepared utilizing antisera previously found active in removing a variety of antigen-specific I-J determinant-bearing SF (5–8). These antisera included B10.D2 anti B10.A (anti-K^k-I-A^k I-B^k I-J^k I-E^k) and B10 anti-B10.A(anti-K^k I-A^k I-B^k I-J^kI$C^d$$S^d$$D^d$). As can be seen in Table VIII, SF passed over these columns were depleted of suppressive activity, which was efficiently recovered in the eluates. Therefore it is apparent that, like many antigen-specific SF, ABA-

TABLE VII

Inbred Strains of Mice Can Make Suppressor Factor Which Limit DTH in the Strain of Origin

Group	Strain	H-2 Haplotype	Heavy-chain linkage group allotype Ig-1	Immunization	Suppressor factor	Mean footpad* increment 10^{-3} in	P
a.							
I	A/J	a	e	3×10^7 ABA/A/J	−	12.7 ± 1.20	—
II	A/J	a	e	3×10^7 ABA/A/J	+	5.1 ± 0.81	<0.005
III	A/J	a	e	—	−	3.0 ± 1.21	<0.001
b.							
I	BALB/c	d	a	3×10^7 ABA-BALB/c	−	18.6 ± 1.57	—
II	BALB/c	d	a	3×10^7 ABA-BALB/c	+	5.9 ± 1.51	<0.001
III	BALB/c	d	a	—	−	3.3 ± 1.78	<0.001
c.							
I	C57BL/6	b	b	3×10^7 ABA-C57BL/6	−	9.4 ± 0.67	—
II	C57BL/6	b	b	3×10^7 ABA-C57BL/6	+	4.4 ± 0.98	<0.005
III	C57BL/6	b	b	—	−	1.2 ± 0.87	<0.005
d.							
I	CBA/J	k	a	3×10^7 ABA-CBA	−	8.5 ± 1.50	—
II	CBA/J	k	a	3×10^7 ABA-CBA	+	2.5 ± 0.88	<0.001
III	CBA/J	k	a	—	−	2.3 ± 0.33	<0.002
e.							
I	B10.A	a	b	3×10^7 ABA-B10.A	−	10.1 ± 0.67	—
II	B10.A	a	b	3×10^7 ABA-B10.A	+	3.1 ± 0.42	
III	B10.A	a	b	—	−	2.0 ± 1.50	
f.							
I	C.AL 20	d	d	3×10^7 ABA-C.AL 20	−	14.5 ± 1.60	
II	C.AL 20	d	d	3×10^7 ABA-C.AL 20	+	5.4 ± 0.75	
III	C.AL 20	d	d	—	−	2.0 ± 1.00	

TABLE VIII

ABA-Cell Induced Suppressor T-Cells-Derived-Suppressor Factors Bear H-2 Region-Encoded Determinants

Group	Suppressor* factor 5×10^8 cell eq	Treatment	Immunization‡	Mean footpad§ increment 10^{-3} in ± SEM	P
I	−	—	3×10^7 ABA A/J	12 ± 1.3	—
II	+	—	3×10^7 ABA A/J	5.4 ± .90	<0.004
III	+	B10αB10.A Seph filtrate	3×10^7 ABA A/J	10.25 ± .80	NS
IV	+	B10αB10.A Seph elution	3×10^7 ABA A/J	6.7 ± 1.3	<0.005
V	+	Normal A/J Ig Seph-filtrate	3×10^7 ABA A/J	5.62 ± 1.2	<0.005
VI	+	B10.D2αB10.A Seph filtrate	3×10^7 ABA	9.8 ± .60	NS
VII	+	B10.D2αB10.A Seph elution	3×10^7 ABA	5.4 ± .97	<0.004
VIII	−	—	—	3 ± 1.1	<0.001

* 2×10^7 suppressor cell equivalents/day per mouse for 5 consecutive d.

‡ 3×10^7 ABA A/J hapten-derivatized cell subcutaneous day 0.

§ 25 μls of active ABA diazonium in the footpad day 5.

induced SF, derived from STC, bear H-2 region-encoded determinants and in particular, those encoded in the K end of H-2.

Discussion

We have previously shown that the generation of antigen-specific contact sensitivity (CS) (12), DTH, or cell-mediated cytotoxicity (CMC) could be elicited by subcutaneous immunization with antigen-derivatized cells (10). We also confirmed the earlier reports of Battisto and Bloom (21) and the later elegant studies of Miller and Claman (11), that the i.v. administration of antigen-derivatized cells stimulated the generation of suppressor T cells which behave in an antigen specific manner (10, 12). In this and the companion paper we have extended these observations to elucidate the molecular nature of this regulatory function.

Suppressor T cells in a variety of systems including that studied by Tada et al. (3, 5), Benacerraf and colleagues (4, 16, 19) and Greene et al. (7, 8), as well as Zembala and Asherson (20), elaborate specific subcellular moieties which can mediate suppression. These SF have in common a number of biochemical characteristics which allow comparisons within this family of biologically active molecules. SF in many cases bear antigen-specific recognition units (5, 7, 8, 19, 20), are proteins derived from the suppressor T cell (5, 9), bear determinants encoded by the I-J subregion of the H-2 MHC (5, 6, 8, 22), and do not bear conventional determinants present on Ig light or heavy chains constant regions (5, 7, 19). The potential mechanisms by which SF manifests suppression include the stimulation and generation of second-order suppressor T cells in as yet an incompletely understood manner (23, 24) and/or alternatively, the direct inactivation of T helper (23) or T-effector cells (20).

In the present series of experiments we have investigated the use of ABA-derivatized cells to induce suppressor cells, and thereafter to obtain SF from these cells. We specifically have chosen the hapten ABA, because A/J mice, when immunized with ABA-KLH, produce a restricted spectrum of anti-ABA antibodies, 20–70% of which bear a specific CRI (13, 18, 25–27). Because the cross-reactive idiotype may be reflective of a germ-line gene product, an analysis of ABA-specific T cells (suppressor, helper, or effector) or the ABA-specific SF might allow structural comparisons to CRI bearing Ig as has been done in other systems (28–30). This comparison is facilitated by the availability of well-characterized anti-idiotype antisera (25, 27, 31–35).

In the present series of experiments, ABA-specific suppressor molecules could be obtained from STC by sonication, snap freezing and thawing and also in the supernates of antigen-stimulated STC. Thus, previously expressed concerns (9) that the differential biological effects of SF may reflect their different modes of isolation may be seen not to apply to the regulatory substances reported herein. ABA cell-induced STC were found in the spleen and, to an extent, in the thymus and were the sources of the SF used in these studies, although lymph node cells have also been found to be suppressive in certain cases (B. A. Bach and M. I. Greene, unpublished observations).

ABA SF are biologically specific in their action inasmuch as only DTH to ABA can be inhibited by the administration of the extract. These biologically specific factors express restricted antigen-binding specificity in that ABA SF interacts only with ABA and not with trinitrophenyl-derivatized cells and will bind to and can be eluted from ABA-FGG immunoadsorbents, indicating specificity for the inducing determinant.

The ABA STC-derived SF can be induced in many strains of mice regardless of H-2 type or Ig-1 allotype. Thus, A/J and C.AL-20, which share the CRI but possess dissimilar H-2 region haplotypes, produce SF which interacts with anti-idiotypic antisera (14). Interestingly, SF produced in A/J mice bear in addition to a genetically coded idiotypic determinant (14), a determinant encoded by genes in the K end of the H-2 MHC. In keeping with the nature of SF in a variety of systems (5–8, 22) it is probable that this determinant is controlled by the I-J subregion; studies are in progress to clarify this point.

Although ABA-specific SF could limit ABA DTH, in the protocol used, anti-ABA antibodies could not inhibit the development of this reactivity. That data taken together with the observed inability of polyvalent rabbit anti-mouse $F(ab')_2$ antisera to interact with SF obviates any consideration that suppressor factor(s) are conventional Ig. Finally, sizing of the SF revealed the greatest suppressive activity in the 33-68,000 peak in accordance with other known T-cell derived regulatory molecules (5–8).

These results and the recent work of Lewis and Goodman (36) establish conclusively that ABA-specific suppressor T cells can be induced. ABA-specific SF which share characteristics of a large family of regulatory molecules with defined immunochemical properties can be obtained from these cells. Whereas SF derived from STC are demonstrably effective in inhibiting CMI, anti-ABA antibody is not, in agreement with previous work. Finally, these studies have permitted the use of ABA SF in a series of experiments outlined in the companion paper which investigates the role of Ig-1-linked genes (14) in the coding of antigen-specific SF.

Summary

Delayed type hypersensitivity to the hapten azobenzenearsonate (ABA) can be induced and suppressed by the administration of hapten-coupled syngeneic spleen cells by the appropriate route.

Suppressor T cells stimulated by the intravenous administration of ABA-coupled spleen cells have been shown to produce a discrete subcellular factor(s) which is capable of suppressing delayed type hypersensitivity to azobenzenearsonate in the mouse. Such suppressor factors may be produced by the mechanical disruption of suppressor cells or by placing such suppressor cells in culture for 24 h. The suppressor factor(s) (SF) derived from ABA-specific suppressor cells exhibit biological specificity for the suppression of ABA delayed type hypersensitivity (DTH), but not trinitrophenyl DTH, as well as the capacity to bind to ABA immunoadsorbents. Passage of suppressor factor(s) over reverse immunoadsorbents utilizing a rabbit anti-mouse $F(ab')_2$ antiserum demonstrated that the antigen-specific T-cell derived SF does not bear conventional immunoglobulin markers. That suppressor factor(s) are not immunoglobulin molecules was further demonstrated by the inability of anti-ABA antibodies to suppress ABA DTH. Gel filtration of ABA suppressor factor(s) showed that the majority of the suppressive activity was present in a fraction with molecular weight ranging between 6.8×10^4 and 3.3×10^4 daltons. We also analyzed for the presence of determinants encoded by the H-2 major histocompatibility complex (MHC) and found that immunoadsorbents prepared utilizing antisera capable of interacting with gene products of the whole or selected gene regions of H-2 MHC, i.e., B10.D2 anti-B10.A and B10 anti-B10.A immunoadsorbents, retained the suppressive

activity of ABA-SF. Elution of such columns with glycine HCl buffers (pH 2.8) permitted recovery of specific suppressive activity.

Taken collectively such data supports the notion that suppressor T-cell-derived ABA suppressor factors have antigen-binding specificity as well as determinants controlled by the K end of the H-2 MHC. The distribution of strains capable of making SF has also been analyzed. The relationship of the antigen-binding specificity to V_H gene products is discussed in this and the companion paper.

We would like to acknowledge the excellent technical assistance of Ms. Cheryl Petrell. We are also grateful to Dr. Masanobu Sugimoto for his comments and early experiments using trinitrophenyl and p-azobenzenearsonate. We are very grateful to Ms. Terri Greenberg for her excellent assistance in the preparation of this manuscript.

Received for publication 15 January 1979.

References

1. Gershon, R. K., and K. Kondo. 1971. Infectious immunological tolerance. *Immunology.* **21:** 903.
2. Asherson, G. L., and M. Zembala. 1976. Suppressor T cells in cell mediated immunity. *Br. Med. Bull.* **32:**158.
3. Tada, T., M. Taniguchi, and T. Takemori. 1975. Properties of primed suppressor T cells and their products. *Transplant. Rev.* **26.**
4. Benacerraf, B., J. A. Kapp, P. Debré, C. W. Pierce, and F. de la Croix. 1975. The stimulation of specific suppressor T cells in genetic non-responder mice by linear random copolymers of L-amino acids. *Transplant. Rev.* **26:**21.
5. Tada, T., M. Taniguchi, and C. S. David. 1976. Properties of the antigen-specific suppressive T-cell factor in the regulation of antibody response of the mouse. IV. Special subregion assignment of the gene(s) that codes for the suppressive T-cell factor in the H-2 histocompatibility complex. *J. Exp. Med.* **144:**713.
6. Thèze, J., C. Waltenbaugh, M. Dorf, and B. Benacerraf. 1977. Immunosuppressive factor(s) specific for L-glutamic acid^{50}-L-tyrosine^{50} (GT). II. Presence of I-J determinants on the GT-suppressive factor. *J. Exp. Med.* **146:**287.
7. Greene, M. I., S. Fujimoto, and A. H. Sehon. 1977. Regulation of the immune response to tumor antigen. III. Characterization of thymic suppressor factor(s) produced by the tumor bearing hosts. *J. Immunol.* **119:**757.
8. Greene, M. I., A. Pierres, M. E. Dorf, and B. Benacerraf. 1977. The I-J subregion codes for determinants on suppressor factor(s) which limit the contact sensitivity response to picryl chloride. *J. Exp. Med.* **146:**293.
9. Kontiainen, S., and M. Feldman. 1978. Suppressor-cell induction in vitro. IV. Target of antigen specific suppressor factor and its genetic relationships. *J. Exp. Med.* **147:**110.
10. Bach, B. A., L. Sherman, B. Benacerraf, and M. I. Greene. 1978. Mechanisms of regulation of cell mediated immunity. II. Induction and suppression of delayed-type hypersensitivity to azobenzenearsonate-coupled syngeneic cells. *J. Immunol.* **121:**1460.
11. Miller, S. D., and H. N. Claman. 1976. The induction of hapten-specific T cell tolerance by using hapten modified lymphoid cells. I. Characteristics of tolerance induction. *J. Immunol.* **117:**1519.
12. Greene, M. I., M. Sugimoto, and B. Benacerraf. 1978. Mechanisms of regulation of cell mediated immune responses. I. Effect of the route of immunization with TNP-coupled syngeneic cells on the induction and suppression of contact sensitivity to picryl chloride. *J. Immunol.* **120:**1604.

13. Nisonoff, A., S-T. Ju, and F. L. Owen. 1977. Studies of structure and immunosuppression of a cross reactive idiotype in strain A mice. *Immunol. Rev.* **34:**89.
14. Bach, B. A., A. Nisonoff, B. Benacerraf, and M. I. Greene. 1979. Mechanisms of Regulation of Cell-mediated Immunity. IV. Azobenzenearsonate specific suppressor factor(s) bear cross-reactive idiotypes determinants, the expression of which is linked to the heavy chain allotype linkage group of genes. *J. Exp. Med.* **149.**
15. Nisonoff, A., W. S. Wissler, and D. L. Woernly. 1959. Mechanisms of formation of univalent fragments of rabbit antibody. *Biochem. Biophys. Res. Commun.* **1, 6:**318.
16. Reif, A. E., and J. M. Allen. 1964. The AKR thymic antigen and its distribution in leukemias and nervous tissues. *J. Exp. Med.* **120:**413.
17. Cuatrecasas, P. 1970. Protein purification by affinity chromatography. *J. Biol. Chem.* **245:** 3059.
18. Ju, S.-T., S. Sato, and A. Nisonoff. 1977. Transfer of idiotype suppression and idiotypes by leukocytes from ascitic fluids. *Eur. J. Immunol.* **7:**401.
19. Kapp, J. A., C. W. Pierce, and B. Benacerraf. 1977. Immunosuppressive factor(s) extracted from lymphoid cells of nonresponder mice primed with L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT). II. Cellular source and effect on responder and nonresponder mice. *J. Exp. Med.* **145:**828.
20. Zembala, M., G. L. Asherson, B. Mayhew, and J. Krejci. 1973. In vitro absorption and molecular weight of specific T-cell suppressor factor. *Nature (Lond.).* **253:**72.
21. Battisto, J. R., and B. R. Bloom. 1966. Dual immunological unresponsiveness induced by cell membrane coupled hapten or antigen. *Nature (Lond.)* **212:**156.
22. Perry, L. L., B. Benacerraf, and M. I. Greene. 1978. Regulation of the immune response to tumor antigens. VI. Tumor antigen specific suppressor factor(s) bear I-J determinants and induce suppressor T cells in vivo. *J. Immunol.* **121:**2144.
23. Tada, T., M. Taniguchi, and T. Tokuhisa. 1978. Suppressive T cell factor and its acceptor expressed on different subsets of T cells: a possible amplification loop in the suppressor system. *In* Proceedings of the Third Ir Gene Workshop, H. O. McDevitt, editor. Academic Press, Inc., N. Y. 517.
24. Germain, R. N., J. Thèze, J. A. Kapp, and B. Benacerraf. 1978. Antigen-specific T-cell-mediated suppression. I. Induction of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} specific suppressor T cells in vitro requires both antigen specific T-cell suppressor factor and antigen. *J. Exp. Med.* **147:**123.
25. Nisonoff, A., and S.-T. Ju. 1976. Studies of a cross reactive idiotype associated with anti-p-azophenylarsonate antibodies in A/J mice. *Ann. Immunol. (Paris).* **127C:**347.
26. Tung, A. S., and A. Nisonoff. 1975. Isolation from individual A/J mice of anti-*p*-azophenylarsonate antibodies bearing a cross-reactive idiotype. *J. Exp. Med.* **141:**112.
27. Kuettner, M. G., A. L. Wang, and A. Nisonoff. 1972. Quantitative investigations of idiotypic antibodies. VI. Idiotypic specificity as a potential genetic marker for the variable regions of mouse immunoglobulin polypeptide chains. *J. Exp. Med.* **135:**579.
28. Cosenza, H., M. H. Julius, and A. A. Augustin. 1977. Idiotypes as variable region markers. Analogies between receptors on phosphorylcholine specific T and B lymphocytes. *Immunol. Rev.* 34:3.
29. Binz, H., H. Wigzell, and H. Bazin. 1976. T cell idiotypes are linked to immunoglobulin heavy chain genes. *Nature (Lond.).* **264:**639.
30. Krammer, P. H., and K. Eichmann. 1977. T cell receptor idiotypes are controlled by genes in the heavy chain linkage group and the major histocompatibility complex. *Nature (Lond.).* **270:**733.
31. Pawlak, L. L., and A. Nisonoff. 1973. Distribution of cross-reactive idiotypic specificity in inbred strains of mice. *J. Exp. Med.* **137:**855.
32. Pawlak, L. L., E. B. Mushinski, A. Nisonoff, and M. Potter. 1973. Evidence for the linkage

of the IgC_H locus to a gene controlling the idiotypic specificity of anti-*p*-azophenylarsonate antibodies in strain A mice. *J. Exp. Med.* **137**:22.

33. Jack, R. S., T. I. Kari, and K. Rajewsky. 1977. Idiotypic analysis of the response of C57BL/6 mice to the (4-hydroxy-3-nitrophenyl) acetyl group. *Eur. J. Immunol.* **8**:559.
34. Eichmann, K. 1975. Genetic control of antibody specificity in the mouse. *Immunogenetics.* **2**: 491.
35. Laskin, J. A., A. Gray, A. Nisonoff, N. R. Klinman, and P. D. Gottlieb. 1977. Segregation at a locus determining an immunoglobulin genetic marker for the light chain variable region affects inheritance of expression of an idiotype. *Proc. Natl. Acad. Sci. U. S. A.* **74**:4600.
36. Lewis, G. K., and J. W. Goodman. 1978. Purification of functional determinant specific idiotype bearing murine T cells. *J. Exp. Med.* **148**:915.