INDUCTION OF T-LYMPHOCYTE RESPONSES TO A SMALL MOLECULAR WEIGHT ANTIGEN

II. Specific Tolerance Induced in Azobenzenearsonate (ABA)-Specific T cells in Guinea Pigs by Administration of Low Doses of an ABA Conjugate of Chloroacetyl Tyrosine in Incomplete Freund's Adjuvant*

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Many studies of specific T-cell tolerance involving the use of small well-defined contact allergens are complicated by the unknown role of autologous protein determinants resulting from in situ conjugation (1–4). Similarly, the multiplicity of determinants present during tolerance induction with foreign proteins hinders suitable analysis of the roles played by different determinants and degradation products that may interact with T-cell subclasses during tolerance induction (5–7). It seemed, therefore, advantageous to develop an experimental model permitting induction of stable T-cell tolerance to a small, well-characterized T-cell immunogen. For this we have selected the low molecular weight compound azobenzenearsonate (ABA)[1] conjugated to N-chloroacetyl tyrosine (ABA-T) and various protein conjugates of this compound. ABA-T and similar derivatives have already proven useful in analysis of hapten-carrier requirements during induction of delayed immunity (8–10), T-cell helper function (11–13), and specific immunological tolerance in newborn (14) and adult guinea pigs (15, 16). In the latter case, however, tolerance induction was of short duration and required substantial amounts of antigen (15, 16).

Previous work in our laboratory has demonstrated that B cells can be optimally tolerized by large doses of polyvalent nonimmunogenic molecules such as dinitrophenyl (DNP) conjugates of the copolymer D-glutamic acid D-lysine (DNP-D-GL) (reviewed in 17 and 18). In contrast, T cells involved in delayed hypersensitivity reactions cannot be tolerized by such molecules since neither

[1] *Abbreviations used in this paper:* ABA, azobenzenearsonate; ABA-T, ABA conjugated to N-chloroacetyl tyrosine; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DNP-D-GL, DNP conjugate of the copolymer of D-glutamic acid and D-lysine; GPA, guinea pig albumin; HGG, human 7s gamma globulin; IFA, incomplete Freund's adjuvant; INS, recrystallized bovine pancreatic insulin; LPS, bacterial lipopolysaccharide; PPD, purified protein derivative of tuberculin.

DNP-D-GL nor ABA-D-GL in large amounts could block delayed hypersensitivity to dinitrochlorobenzene or ABA-T, respectively (reference 19 and footnote 2). It was therefore suggested that acquired T-cell tolerance may be an active process which should be inducible with "immunogenic doses" of tolerogen administered under non-immunogenic conditions.[2]

The experiments presented in this paper demonstrate that the induction of tolerance on the one hand and the induction of delayed sensitivity on the other hand can be accomplished by administration of similar doses of ABA-T to guinea pigs with the determining factor being the absence or presence, respectively, of activating bacterial products in the adjuvant mixture used. Thus, complete, persistent ABA-T-specific T-cell tolerance can be induced in adult guinea pigs with 20 μg of ABA-T given intradermally in incomplete Freund's adjuvant (IFA), whereas this same dose of ABA-T induces ABA-specific immunity when administered in complete Freund's adjuvant (CFA). This tolerance was not reversible by administration of ABA-T and IFA in the presence of bacterial lipopolysaccharide (LPS), was generated before the formation of primed T cells, and persisted for at least 3 mo after initiation.

Materials and Methods

Proteins and Chemical Reagents. Bovine serum albumin (BSA) and guinea pig albumin (GPA) were purchased from Pentex Biochemical, Kankakee, Ill. Recrystallized bovine pancreatic insulin (INS) was obtained from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. Human 7S gamma globulin (HGG), chloroacetyl-L-tyrosine, and tyramine hydrochloride were purchased from Mann Research Labs., Inc., Springfield, Mo. *Escherichia coli* LPS (055:B5) was obtained from Difco Laboratories, Inc., Detroit, Mich. (lot no. 585002).

Antigens

PREPARATION OF MONO(*p*-AZOBENZENEARSONIC ACID)-*N*-CHLOROACETYL-L-TYROSINE (ABA-T). 500 mg *p*-aminobenzenearsonic acid in 20 ml distilled H_2O was dissolved by addition of 3 ml of 1N HCl, placed on ice and then 3 ml of 5% $NaNO_2$ was added to form the diazonium salt. 1 g of chloroacetyl-L-tyrosine was dissolved in 20 ml of 5% Na_2CO_3 and the pH adjusted to 9 with 1N NaOH. The cold diazonium salt of arsanilate was slowly added to the chloroacetyl-L-tyrosine solution and the mixture stirred at room temperature for 2 h maintaining the pH at 9 with 1N NaOH. The ABA-T product was precipitated by acidification with 1N HCl and washed. The compound was redissolved and reprecipitated twice from water by bringing the pH to 9 and then acidifying slowly. The concentration of ABA-T was determined spectrophotometrically using $E_{1\,cm} - 10,500$ at 490 nm in 0.1 N NaOH (20, 21).

PREPARATION OF ABA-PROTEIN CONJUGATES. Proteins were reacted with the diazonium salt of arsanilate for 3 h at room temperature using ratios of 1:2 or 1:6 (milligrams arsanilate:milligrams protein) for high and low substitutions, respectively. The resulting conjugates were dialyzed extensively against phosphate-buffered saline (PBS) at 4°C. Final protein concentrations were determined by micro-Kjeldahl analysis (22), and the degree of ABA substitution of tyrosine residues was determined spectrophotometrically. Since ABA-lysine does not absorb at 490 nm (21), it was assumed that the degree of lysine substitution did not significantly affect these calculations. The ABA molar substitution ratios were: ABA_{49}-HGG, ABA_{20}-BSA, ABA_3-GPA, and ABA_4-INS.

Animals and Immunizations. Male Hartley and strain 13 guinea pigs, 300–600 g, were obtained

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[2] Bullock, W. W., D. H. Katz, and B. Benacerraf. 1975. Induction of T-lymphocyte responses to a small molecular weight antigen. I. Failure to induce tolerance in azobenzenearsonate (ABA)-specific T cells in guinea pigs with ABA conjugate of a copolymer of D-glutamic acid and D-lysine. *J. Immunol.* **115:** in press.

from Camm Research Institute, Inc., Wayne, N. J. Antigens were dissolved in 0.15 M NaCl and emulsified with an equal volume of either CFA or IFA (Difco Laboratories, Inc.). For CFA immunization of strain 13 guinea pigs, IFA was mixed with *Mycobacterium tuberculosis* H37RV (2.5–5.0 mg/ml). For immunization, 0.4 ml was equally distributed into the four foot pads or at four intradermal neck sites.

Skin Testing. Animals were shaved along each side and tested at one to four sites. Intradermal injections consisted of 10 or 50 μg of antigen given in 0.1 ml of saline. Reactions were read at 3, 24, and 48 h. The diameter (millimeters) of the erythema and degree of induration were recorded at 24 and 48 h.

Statistical Analysis. Means and standard errors of the diameters of delayed hypersensitivity reactions of the various experimental groups were calculated arithmetically and group comparisons were made employing Student's t test.

Results

Determination of Optimal Immunogenic Dose Range of ABA-T in Random Bred and Inbred Guinea Pigs. Groups of random bred Hartley guinea pigs were immunized with log incremental doses of ABA-T in CFA (2–2,000 μg/animal) or with CFA alone. 2 wk later all animals were skin tested with ABA-HGG and purified protein derivative of tuberculin (PPD). As shown in Table I, doses of 20

TABLE I
Dose Response for Induction of Delayed Sensitivity with ABA-T Administered in CFA

Protocol*		Delayed skin reactions‡	
Group	Immunization (wk 0)	ABA-HGG (50 μg, wk 2)	PPD (10 μg, wk 2)
		mm	*mm*
A	Saline (CFA)	4.0 (±0.4)	23.2 (±0.9)
B	2 μg ABA-T (CFA)	6.0 (±0.7)	23.7 (±1.0)
C	20 μg ABA-T (CFA)	19.5 (±0.6)	24.0 (±0.6)
D	200 μg ABA-T (CFA)	26.3 (±3.3)	22.5 (±1.6)
E	2,000 μg ABA-T (CFA)	19.0 (±1.6)	22.2 (±1.0)

* Hartley guinea pigs were immunized with log incremental doses of ABA-T administered in CFA in the foot pads. 2 wk later, these animals were skin tested with ABA-HGG and PPD.

‡ Skin tests were performed on individual animals in each group using 50 μg ABA-HGG and 10 μg PPD. The data are presented as the average reaction diameters in millimeters of groups of four guinea pigs recorded 24 h after intradermal sensitization. Numbers in parentheses are standard errors.

and 200 μg of ABA-T were optimal for eliciting ABA-specific T-cell immunity; the 2 μg dose of the range tested (2–2,000 μg) was clearly less effective. Immunization with CFA alone failed, as expected, to induce sensitivity to ABA-T, although good sensitivity to PPD was obtained in this group. Studies with inbred strain 13 guinea pigs demonstrated comparable responses with the optimal doses, provided that H37RV *Mycobacterium* was used in the CFA.

Induction of Transient ABA-Specific Tolerance in Guinea Pigs after Intravenous Administration of ABA-T. In previous studies of Leskowitz and Collotti (15, 16), ABA-specific tolerance was induced by intracardiac or intraperitoneal administration of aqueous ABA-T before sensitization with immunogenic quantities of ABA-T in CFA. The data presented in Table II summarizes our own

Table II

Induction of Transient ABA-Specific Tolerance in Guinea Pigs after Intravenous
Administration of ABA-T

	Protocol*		Delayed skin reactions‡	
			ABA-BSA (50 μg, wk 3)	ABA-HGG (50 μg, wk 7)
Group	Pretreatment (wk 0)	Immunization		
			mm	*mm*
A	None	50 μg ABA-T (CFA)	16.6 (±5.2)	14.7 (±2.7)
B	2.0 mg ABA-T intravenously	50 μg ABA-T (CFA)	2.6 (±0.2)	10.5 (±2.0)

* Hartley guinea pigs were pretreated with 2.0 mg ABA-T administered intravenously (group B). 30 min later this group plus an additional group were sensitized with 50 μg ABA-T in CFA in the foot pads. All animals plus a group of unsensitized controls were skin tested at 3 wk and 7 wk after immunization with ABA-BSA and ABA-HGG, respectively.

‡ Skin tests were performed on individual animals in each group using 50 μg of the test antigen indicated. The data are presented as the average reaction diameters in millimeters of groups of five guinea pigs recorded 24 h after intradermal sensitization. All unsensitized control reactions were <2 mm. Numbers in parentheses are standard errors. Statistical analysis of mean delayed skin reactions yielded P values of <0.001 at 3 wk and >0.2 at 7 wk for group B compared to group A.

experience with such attempts at tolerance induction in this system. Guinea pigs were injected intravenously with either 2.0 mg of aqueous ABA-T or, as controls, with saline and then immunized with 50 μg of ABA-T in CFA immediately thereafter. Skin tests performed with ABA-BSA 3 wk after sensitization revealed a substantial suppression of ABA-specific delayed hypersensitivity in the group pretreated with intravenous ABA-T as compared to controls. The PPD responses were equivalent in the two groups (not shown). The suppression of ABA-specific responses in this system was only transient, however, since by wk 7 after sensitization, the level of ABA-specific delayed hypersensitivity was essentially indistinguishable between the treated and untreated groups.

Suppression of ABA-Specific T-Cell Responses by Administration of Immunogenic Doses of ABA-T in IFA. In the preceding experiment it was shown that intravenous administration of ABA-T induced a transient state of specific unresponsiveness. The transient nature of the unresponsiveness induced in this manner is perhaps not surprising in view of the previous demonstration by Leskowitz that 95% of the ABA-T is excreted within 5 days after intravenous injection (15). In order to analyze the mechanism(s) underlying T-cell tolerance, we felt that a regimen for inducing tolerance of longer duration with a moderate-to-low dose of ABA-T would be desirable. This was accomplished by administering ABA-T as a depot in IFA emulsions.

As shown in Fig. 1, groups of Hartley guinea pigs were injected with log incremental doses (2-200 μg) of ABA-T in IFA in the foot pads, and 1 wk thereafter these animals and a group of untreated controls were immunized with 50 μg of ABA-T in CFA intracutaneously. 4 wk later all animals were skin tested with ABA-HGG and PPD. The guinea pigs injected with ABA-T in IFA were in all instances suppressed in responsiveness to ABA-T as compared to untreated
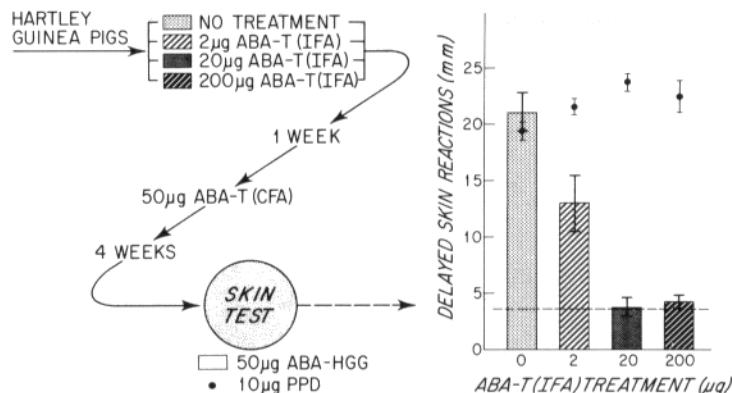
FIG. 1. Hartley guinea pigs were pretreated with log incremental doses (2–200 μg) of ABA-T in IFA in the foot pads. 1 wk later these animals and a group of untreated controls were intracutaneously immunized with 50 μg of ABA-T in CFA. 4 wk later all animals were skin tested with 50 μg of ABA-HGG and 10 μg of PPD. The data are presented as the average reaction diameters in millimeters of groups of six guinea pigs each recorded 24 h after intradermal sensitization. The vertical lines represent standard errors. The dashed line represents the mean reaction diameter of nonsensitized control guinea pigs skin tested with the same preparation of ABA-HGG at the same time. Statistical comparisons of the mean skin reactions to ABA-HGG of the pretreated groups with those of the untreated controls yielded the following P values: $P = 0.035$ for the group pretreated with 2 μg, and $P < 0.001$ for the groups pretreated with 20 and 200 μg.

controls. The optimal doses resulting in suppression were 20 and 200 μg but even the lowest of 2 μg exerted a detectable suppressive effect. The unresponsiveness was ABA-specific since the degree of PPD sensitivity was not diminished in any of the pretreated animals (indeed, PPD reactivity was inversely related in magnitude to ABA-specific responses). Moreover, pretreatment with IFA alone did not diminish responsiveness to subsequent immunization with ABA-T in CFA (not shown).

The suppressive effect of pretreatment with ABA-T in IFA appears to be relatively persistent. In the experiment shown in Fig. 2, two groups of guinea pigs were injected with 50 μg of ABA-T in either CFA or IFA; 2 wk later these animals and a third group of untreated guinea pigs were skin tested with ABA-HGG followed by intradermal immunization with ABA-T in CFA. As shown, it is clear that only the group of guinea pigs sensitized initially with ABA-T in CFA manifested ABA-specific delayed hypersensitivity at this point (wk 2). A second skin test performed with ABA-BSA 2 wk later (wk 4 of the experiment) revealed positive ABA-specific reactions in all but the guinea pigs that had been initially treated with ABA-T in IFA. The significant diminution at wk 4 in the magnitude of responses in the group sensitized with ABA-T in CFA at the beginning of the experiment and then immunized again with ABA-T in CFA at wk 2 may reflect the use of the same mycobacterial adjuvant within a short interval, but, in fact, is without a definitive explanation at this time. Even as late as 7 wk after initiation of the experiment, guinea pgis pretreated with ABA-T in IFA were significantly suppressed in their capacity to develop ABA-specific responses as
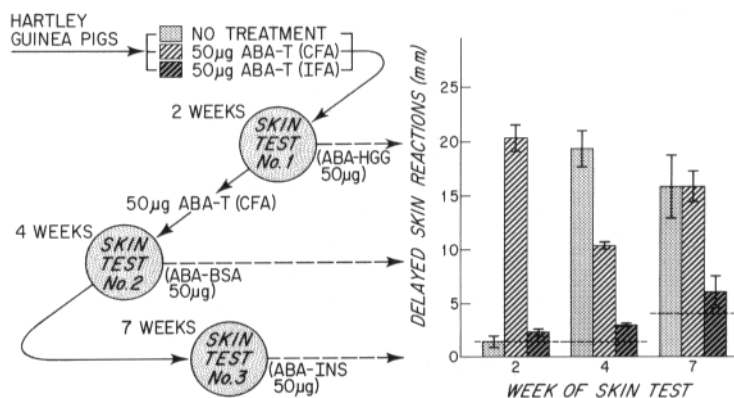
FIG. 2. Guinea pigs were injected with 50 μg of ABA-T in either CFA or IFA; 2 wk later these animals and a third group of untreated guinea pigs were skin tested with ABA-HGG followed by intradermal immunization with 50 μg of ABA-T in CFA. 2 wk later (wk 4 of the experiment) animals were skin tested with 50 μg of ABA-BSA. A third skin test was performed with 50 μg of ABA-INS 7 wk after initiation of the experiment. The data are presented as the average reaction diameters in millimeters of groups of three guinea pigs recorded 24 h after intradermal sensitization. The vertical lines represent standard errors. The dashed lines represent the mean reaction diameters of nonsensitized control guinea pigs skin tested with the same preparations of ABA-HGG, ABA-BSA, and ABA-INS at each time in the experiment. Statistical comparisons of the mean skin reactions of the groups treated at the initiation of the experiment with the untreated controls yielded the following P values at the various times of skin tests: (a) wk 2, $P < 0.001$ in the case of ABA-T (CFA) and $P > 0.90$ in the case of ABA-T (IFA); (b) wk 4, $P = 0.007$ in the case of ABA-T (CFA) and $P < 0.001$ in the case of ABA-T (IFA); (c) wk 7, $P > 0.90$ in the case of ABA-T (CFA) and $P = 0.004$ in the case of ABA-T (IFA). Comparison of the reactions at wk 4 of the group initially sensitized with ABA-T in CFA with those given ABA-T in IFA yielded $P < 0.001$.

evidenced by a third skin test elicited with ABA-INS. Again, the suppression was specific since PPD responses were not appreciably different between the groups (not shown). More recent experiments have shown this tolerance to persist for greater than 3 mo.

The administration of ABA-T in IFA may exert a suppressive effect on either the initial T-cell sensitization phase or block the elicitation of delayed skin reactivity. In order to distinguish between these two possibilities, an experiment was performed to determine whether or not treatment with ABA-T in IFA would exert any suppressive effect in animals that had been previously sensitized with ABA-T in CFA and then skin-tested 1 wk later with ABA-BSA. As shown in Fig. 3, two groups of guinea pigs so sensitized displayed ABA-specific delayed responses 7 days later. At this time, one group was treated with intradermal injections of ABA-T in IFA in an attempt to abrogate the sensitive state. However, as shown by subsequent skin tests on days 18 and 31, no significant depression of the ABA-specific delayed responses was obtained as a result of such treatment. These data indicate, therefore, that previously sensitized T cells cannot be rendered unresponsive by this regime, and also that the administration of ABA-T in IFA does not inhibit elicitation of skin tests in appropriately sensitized animals.

The Effects of LPS on the Suppressive Properties of ABA-T Administered in IFA. The potent tolerogenic or suppressive effects of ABA-T administered in IFA contrasts sharply with the very immunogenic properties of this antigen when incorporated in complete adjuvant containing mycobacterial products. Since previous studies in our laboratory (23–25) and others (26) have demonstrated that LPS can exert marked adjuvant effects on T-lymphocyte functions, we sought to determine whether incorporation of LPS into the IFA emulsion would influence in any way the suppression phenomenon normally resulting from administration of ABA-T in this manner. Groups of guinea pigs were injected with 50 µg of ABA-T incorporated, respectively, into emulsions of IFA alone, IFA containing 200 µg of LPS, CFA alone, and CFA containing 200 µg of LPS. Skin tests for ABA-specific delayed sensitivity responses 2 wk later again demonstrated marked suppression in the group that had received ABA-T in IFA as compared to guinea pigs sensitized with ABA-T in CFA (Table III). More significantly, the incorporation of LPS into the IFA emulsion completely reversed the tolerogenic properties and resulted in ABA-specific delayed responses that were comparable to those obtained in animals immunized with ABA-T in CFA. The addition of LPS to CFA had no appreciable influence on the magnitude of ABA sensitivity elicited in this way. The capacity of LPS to convert ABA-T in IFA to an immunogenic rather than tolerogenic emulsion was not merely a transient effect since skin tests performed 5 wk later also revealed differences comparable to those observed at 2 wk (data not shown).

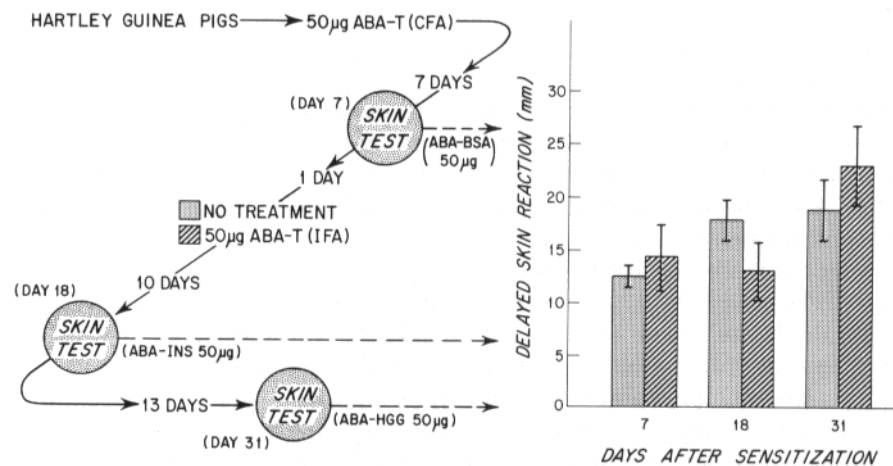A final experiment was performed to determine whether the potent adjuvant

FIG. 3.   Two groups of animals were immunized with 50 µg ABA-T in CFA in the foot pads. 7 days later each group was skin tested with 50 µg ABA-BSA. 1 day later one group intradermally received 50 µg of ABA-T in IFA; the second group was not treated. On days 18 and 31 after initial sensitization, both groups were again skin tested with 50 µg of either ABA-INS or ABA-HGG. The data are presented as the average reaction diameters in millimeters of groups of five guinea pigs recorded 24 h after intradermal sensitization. The vertical lines represent standard errors. Statistical comparisons of the mean skin reactions of the two groups yielded *P* values of *P* > 0.20 or greater at each day tested.

TABLE III
The Effects of LPS on the Suppressive Properties of ABA-T Administered in IFA

Protocol*		Delayed skin reactions‡ with ABA-HGG (50 μg, wk 2)
Group	Immunization (wk 0)	
		mm
A	50 μg ABA-T (IFA)	2.3 (±0.4)
B	50 μg ABA-T (IFA + LPS)	19.7 (±0.9)
C	50 μg ABA-T (CFA)	20.3 (±1.2)
D	50 μg ABA-T (CFA + LPS)	17.7 (±0.9)

* Hartley guinea pigs were injected in the foot pads with 50 μg ABA-T either in IFA alone, IFA plus 200 μg LPS, CFA alone, or CFA plus 200 μg LPS. 2 wk later all animals were skin tested with ABA-HGG.

‡ Skin tests were performed on individual animals in each group using 50 μg of ABA-HGG. The data are presented as the average reaction diameters in millimeters of groups of three guinea pigs recorded 24 h after intradermal sensitization. Numbers in parentheses are standard errors. Statistical comparisons of the mean skin reactions to ABA-HGG yielded P values of >0.15 comparing group B and D to C and <0.001 comparing group A to C.

influence of LPS could be utilized to perhaps abrogate a previously induced state of ABA-specific unresponsiveness. Two groups of guinea pigs were pretreated with 50 μg of ABA-T in IFA. 3 wk later these animals and two groups of untreated controls were sensitized, respectively, with 50 μg of ABA-T either in CFA or IFA containing 200 μg of LPS/animal. As shown in Fig. 4, skin tests performed 2 wk after sensitization revealed good delayed hypersensitivity responses in the two untreated control groups which were comparable in magnitude for both immunization regimens. In contrast, guinea pigs pretreated with ABA-T in IFA failed to develop ABA-specific responses irrespective of whether CFA or IFA plus LPS had been used for sensitization.

Passive Transfer of ABA-Specific Suppression with Lymphoid Cells from Donors Pretrated with ABA-T in IFA.   The capacity of ABA-T administered in IFA in moderate doses to result in markedly diminished responsiveness to subsequent immunization with ABA-T in CFA suggests the possibility of an active suppression phenomenon mediated by lymphoid cells. This possibility was tested in a cell transfer experiment in the following manner: Inbred strain 13 guinea pigs were immunized with 50 μg of ABA-T in CFA or injected with 50 μg of ABA-T in IFA. 3 wk later these respective donor groups were killed and their axillary, occipital, inguinal, and popliteal lymph nodes and spleens removed. Single cell suspensions, in minimum essential medium (Eagle's) were prepared, washed, and transferred intravenously to unprimed, syngeneic recipients (400 × 10⁶ cells/recipient). 1 wk after cell transfer these two groups and a third group of control guinea pigs were skin tested with 60 μg ABA-BSA. Positive skin reactions were manifested only by the group that had received cells from donors
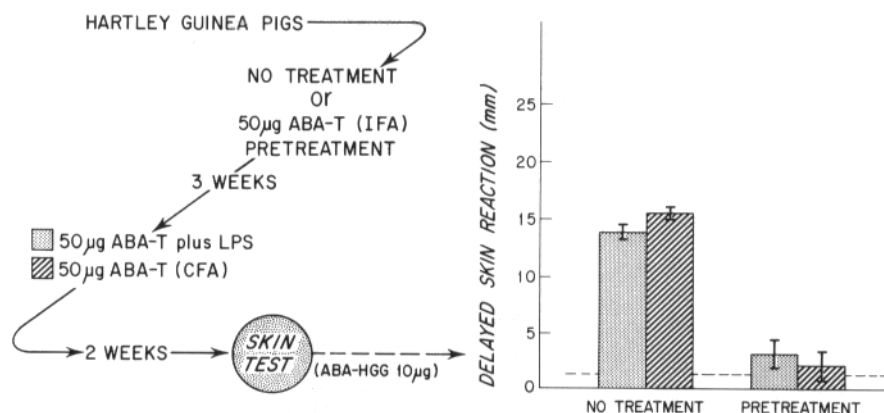
FIG. 4. Two groups of guinea pigs were pretreated with 50 μg of ABA-T in IFA. 3 wk later these animals and two groups of untreated controls were sensitized, respectively, with 50 μg of ABA-T in either CFA or IFA containing 200 μg LPS/animal. Skin tests were performed 2 wk later with 10 μg of ABA-HGG. The data are presented as the average reaction diameters in millimeters of groups of five guinea pigs recorded 24 h after intradermal sensitization. The vertical lines represent standard errors. The dashed line represents the mean reaction diameters of nonsensitized control guinea pigs skin tested with the same preparation of ABA-HGG at the same time. Statistical comparisons of the mean skin reactions of the pretreated groups with those of the untreated controls yielded P values of $P < 0.001$ in all cases.

immunized with ABA-T in CFA (data not shown). 1 day after skin tests all animals were immunized with 50 μg of ABA-T in CFA. 2 and 5 wk later all three groups were tested for delayed sensitivity to ABA and PPD. At 2 wk both groups of cell transfer receipients were partially suppressed in their responsiveness to both ABA and PPD in comparison to the control group indicating some form of nonspecific suppression. 5 wk after immunization (Fig. 5), however, it was clear that recipients of cells from donors immunized with ABA-T in CFA responded to ABA comparably to the control animals, whereas recipients of cells from donors injected with ABA-T in IFA were markedly suppressed in this regard. Although the magnitude of PPD reactivity in the latter group was somewhat lower than the other two groups, the difference was not significant.

Discussion

The studies presented in this paper demonstrate that the administration of small-to-moderate doses of ABA-T in IFA to unprimed guinea pigs induces a state of ABA-specific unresponsiveness as evidenced by the failure of animals so treated to respond to immunization with ABA-T in CFA. The unresponsiveness induced in this manner is persistent, indeed considerably more so than the transient unresponsiveness induced by intravenous administration of large doses of ABA-T as shown in earlier studies of Leskowitz and Collotti (15, 16) and in the present studies. In previous studies, Loewi et al. were successful in inducing tolerance to the mucopolysaccharide blood group substance A by administering the latter in IFA to guinea pigs 2 wk before immunization with the substance in
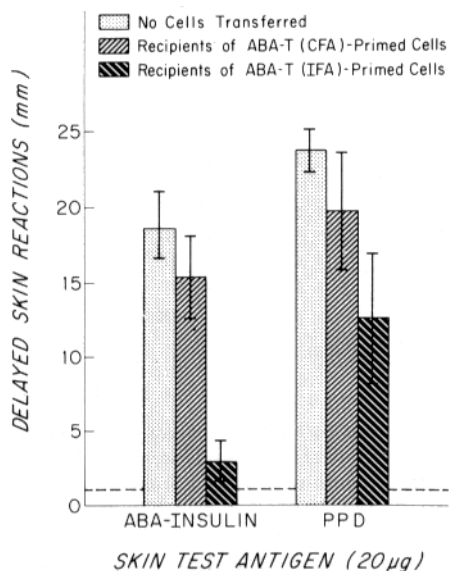
FIG. 5. Passive transfer of ABA-specific suppression with lymphoid cells from donors pretreated with ABA-T in IFA. Inbred strain 13 guinea pigs were immunized with 50 μg of ABA-T in CFA or injected with 50 μg of ABA-T in IFA. 3 wk later these respective donor groups were killed and their axillary, occipital, inguinal, and popliteal lymph nodes and spleens removed. Single cell suspensions in minimum essential medium (Eagle's) were prepared, washed, and transferred intravenously to unprimed, syngeneic recipients (400 × 10⁶ cells/recipient). 1 wk after cell transfer, these two groups (five recipients each) and a third group of three control guinea pigs were skin tested with 60 μg ABA-BSA. 24 h later all animals were immunized in the foot pads with 50 μg of ABA-T in CFA. 2 and 5 wk later all three groups were tested for delayed sensitivity to ABA and PPD. The data presented are the average reaction diameters in millimeters of the various groups recorded 24 h after skin testing with the test antigens indicated at wk 5 after immunization. The vertical lines represent standard errors. The dashed line represents the mean reaction diameters of nonsensitized control guinea pigs skin tested with the same preparations of ABA-INS and PPD at the same time. Statistical comparisons of the mean skin reactions of the two groups of cell transfer recipients with the guinea pigs that did not receive cell transfers yielded the following P values for the two skin-test antigens: (a) ABA-INS, $P = 0.42$ and $P = 0.0015$ for recipients of CFA-primed and IFA-primed cells, respectively; (b) PPD, $P = 0.49$ and $P = 0.093$ for recipients of CFA-primed and IFA-primed cells, respectively. Statistical comparisons of the reactions of the two cell transfer recipient groups yielded P values of 0.006 and 0.28 for ABA-INS and PPD, respectively.

CFA (27). The tolerance to ABA-T described in this paper is specific since T-cell responses to PPD were not diminished by administering ABA-T in IFA before subsequent sensitization with CFA.

The tolerant state induced by ABA-T in IFA has been shown to require administration of ABA-T in the absence of bacterial adjuvant products. Thus, incorporation of LPS into the IFA emulsion resulted in the development of delayed hypersensitivity to ABA-T comparable in strength to that obtained with ABA-T in CFA. The capacity of LPS to have this effect is another demonstration of the strong adjuvant properties of this substance on T-lymphocyte functions.

Previous studies from this laboratory have demonstrated that LPS, in addition to its mitogenic activity on B lymphocytes, is highly effective in regulating T-cell functions such as the development and function of carrier-specific helper cells in humoral responses (23–25). The present studies show that LPS is capable of regulating functions of T cells that are involved in development of delayed hypersensitivity responses. Rcently, Nauciel et al. reported that whole gram-negative bacteria, endotoxin, and purified cell wall peptidoglycan were all capable of acting as adjuvant when added to ABA-acetyl-tyrosine IFA emulsions (28). However, further study will be required to determine if both peptidoglycan and LPS are effective adjuvants or whether minor contamination of LPS by peptidoglycan is the cause of the observed LPS activity.

The possibility that the unresponsiveness resulting from ABA-T in IFA reflected a phenomenon of active specific suppression was suggested by the fact that in the present studies only relatively modest doses were required to induce a rather persistent state of tolerance, and by the previous observations of Collotti and Leskowitz (16) demonstrating that only ABA derivatives of immunogenic carriers were capable of inducing ABA-specific tolerance. Indeed, the passive transfer of lymph node and spleen cells from guinea pig donors pretreated with ABA-T in IFA significantly suppressed the subsequent responses of unprimed syngeneic recipients of such cells to sensitization with ABA-T in CFA. Moreover, in the accompanying manuscript, we will present evidence demonstrating that animals rendered unresponsive by ABA-T in IFA possess ABA-specific suppressor cells which are capable of suppressing delayed sensitivity responses to determinants on proteins covalently linked to ABA (29). These findings, although demonstrating the participation of active suppressor cells in this model, do not define the class of cell responsible for the suppression. We are assuming that this suppression is mediated by specific T lymphocytes since the administration of ABA-T generally fails to induce ABA-specific B-cell responses (9–12). On the other hand, the recent indication of the participation of "suppressor" B cells in other studies performed in guinea pigs (30, 31) leaves this an open possibility in the ABA system that will require further delineation.

It is pertinent that the capacity of ABA-specific suppressor cells to induce and maintain a state of unresponsiveness could only be demonstrated in unprimed animals and likewise that the unresponsive state was not reversed by administration of ABA-T in the presence of LPS. Both of these observations point to the likelihood that the cellular locus of suppression may well be that T lymphocyte that will perform a helper function in the generation of ABA-specific effector T cells. This reasoning follows from the assumption that if ABA-specific suppression were mediated directly on the mature effector cells, then one would expect to observe suppression in either the inductive or elicitation phases of subsequent immunization and skin testing of previously primed animals by administering ABA-T in IFA. Such was not the case, however (Fig. 3). Moreover, since the adjuvant properties of LPS and similar agents appear to exert most of their effects on functions of helper or regulator T lymphocytes (23–27), the failure of LPS to reverse unresponsiveness in this system is expected if the suppressor cells were acting to inhibit or diminish the numbers and/or activity of such helper T cells. This is precisely what was observed in these studies (Fig. 4).

Further delineation of this point is extremely important in view of the critical impact this might have on the therapeutic potential of such models of immune suppression. If this specific suppression has little capacity to regulate the responses of fully mature, primed effector cells, the clinical usefulness of this approach would be severely limited.

Summary

The experiments presented in this paper demonstrate that the induction of tolerance on the one hand and the induction of delayed sensitivity on the other hand can be accomplished by administration of similar doses of azobenzene-arsonate conjugated to N-chloroacetyl tyrosine (ABA-T) to guinea pigs with the determining factor being the absence or presence, respectively, of activating bacterial products in the adjuvant mixture used. Thus, complete, persistent ABA-T-specific T-cell tolerance can be induced in adult guinea pigs with 20 μg of ABA-T given intradermally in incomplete Freund's adjuvant (IFA) whereas this same dose of ABA-T induces ABA-specific immunity when administered in complete Freund's adjuvant. This tolerance was not reversible by administration of ABA-T and IFA in the presence of bacterial lipopolysaccharide, was generated before the formation of primed T cells, and persisted for at least 3 mo after initiation. Morover, cell transfer studies performed herein demonstrate that the unresponsiveness resulting from administration of ABA-T in IFA reflects the activity of ABA-specific suppressor cells. The findings that the capacity of ABA-specific suppressor cells to induce and maintain a state of unresponsiveness could only be demonstrated in unprimed animals may indicate a severe limitation on the potential clinical usefulness of such an approach to regulation of the immune system.

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