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Production of Hapten-Specific Unresponsiveness in Adult Guinea-Pigs by Prior Injection of Monovalent Conjugates

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Summary. Adult guinea-pigs injected with a small monovalent conjugate of arsanilic acid, from 2 weeks before to the day of immunization, with hapten-conjugate in adjuvant show a profound depression of hapten-specific delayed sensitivity. The same conjugate given 1–2 weeks after immunization is no longer effective. More than 95 per cent of the intravenously administered monovalent conjugate was found to be excreted in 5 days. Passively administered antibody was without effect on the subsequent development of the hapten-specific delayed sensitivity. These observations are consistent with the hypothesis that the non-immunizing intravenous injection of monovalent conjugate is paralysing to precursor cells if given first, but that once these cells are engaged by adjuvant immunization they are no longer repressible but only transiently inhibitable, as long as high concentrations of monovalent conjugate remain in the circulation.

INTRODUCTION

The production of immunological unresponsiveness by injection of the antigen into neonates is now a classic and easily reproducible phenomenon. The essential elements for production of such unresponsiveness have been considered to be: (a) a large amount of antigen relative to the amount of lymphoid tissue present; (b) persistence of antigen; and (c) relative simplicity in terms of antigenic determinants in the antigen involved (Smith, 1961). Production of similar unresponsiveness by injection of antigen into adult animals has, with the exception of Felton's 'immunological paralysis' of mice to pneumococcal polysaccharide (Felton, 1949), been relatively difficult to achieve. Thus, massive infusions of protein antigens were required to produce transient unresponsiveness in adult rabbits (Dixon and Maurer, 1955) and guinea-pigs (Liacopoulos and Neveu, 1964).

Many years ago, Sulzberger (1929) demonstrated that an intravenous injection of neoarsphenamine into adult guinea-pigs prevented subsequent contact sensitization with this compound. This work has been extended to other contact allergens, such as picryl chloride, where it has been shown that feeding (Chase, 1946) or injection into the mesenteric vein (Battista and Miller, 1962) has produced unresponsive states in adult guinea-pigs.

Recently, a number of publications have appeared demonstrating that injections of relatively small quantities of antigen prior to immunization with Freund's adjuvant produced a profound immunological suppression in mice (Dresser, 1962; Dietrich and Weigle, 1964). This suppression was in respect to antibody formation.

Similar experiments by Asherson and Stone (1965) with guinea-pigs demonstrated a suppression of delayed sensitivity and a change in antibody production which led the authors to designate this phenomenon 'immune deviation'. However, a parallel study by Dvorak, Billote, McCarthy and Flax (1965) showed that when the appropriate dose of antigen was used, the unresponsiveness produced extended to the various immunoglobulins as well as to delayed sensitivity.

In our past studies on hapten-specific delayed hypersensitivity, we were struck by the observation that injection of a variety of monovalent conjugates of arsanilic acid into newborn guinea-pigs produced a persistent unresponsiveness in regard to such delayed sensitivity (Jones and Leskowitz, 1965a). Injection of similar conjugates into adult sensitized guinea-pigs produced a prolonged state of desensitization if multivalent conjugates were used, but only transient suppression of delayed sensitivity if monovalent conjugates were used (Leskowitz and Jones, 1965). Because it appeared that monovalent conjugates of arsanilic acid produced strikingly different effects when injected at birth and when injected post-sensitization, it was decided to re-explore this phenomenon to determine its relationship to the type of unresponsiveness shown by others to follow injections of soluble antigen prior to immunization with adjuvant.

MATERIALS AND METHODS

Antigens

All the conjugates used for immunization and skin testing were prepared as previously described (Jones and Leskowitz, 1965a) by diazotizing the appropriate amount of arsanilic acid in ice-cold solution and coupling the diazonium compounds overnight at pH 8–9 in the cold with the required amounts of protein or *N*-acetyltyrosine. Protein conjugates were purified by dialysis against cold running water, the azobenzenearsonate conjugate of *N*-acetyltyrosine (ABA–tyr) by repeated precipitation following acidification.

Animals

Albino random-bred male guinea-pigs (400 g) were used throughout.

Immunization and skin-testing

Antigens for immunization were emulsified with an equal volume of complete Freund's adjuvant (mineral oil 8·5, Arlacel 1·5, mycobacteria 5 mg/ml). A total volume of 0·1 ml of emulsion was distributed amongst the four footpads. Two weeks later, guinea-pigs were shaved, depilated with Nair (Carter Products, Inc., New York) and skin-tested with the appropriate antigens contained in a volume of 0·1 ml. Reactions were looked at 3 hours later for evidence of Arthus reaction and then measured with a millimetre ruler at 24 hours for extent of induration and erythema.

Tritium-labelled arsanilic acid conjugates ($[^3H]ABA$)

Arsanilic acid tritiated by catalytic exchange (New England Nuclear Corporation, Boston, 3·8 c/mg) was diazotized and coupled to *N*-acetyltyrosine in the usual way. After purification by repeated precipitation in acid and dissolving in alkali, a sample was diluted with carrier ABA–tyr and analysed electrophoretically on cellulose acetate paper. A very small amount of more rapidly migrating purplish material was seen, which on scintillation scanning was found to comprise perhaps 5 per cent of total radioactivity. In

all cases a maximum of 33 per cent of the label appeared to conjugate to the carrier. The rest of the labelled material was presumed to be degraded arsanilic acid incapable of diazotization.

For clearance studies, 300-g guinea-pigs were lightly anaesthetized with ether and the required dose of [^{3}H]ABA–tyr in saline injected by intracardiac or intraperitoneal routes. The syringe was rinsed several times with blood before withdrawal from the heart.

The guinea-pigs were placed in metabolism cages, and the collected urine and faeces removed at intervals for assay. At 1 week, animals were killed and various organs removed and weighed. Portions of the various tissues were ground with water in a motor-driven Elvejhem–Potter mill. Aliquots were dissolved in hyamine (Packard Institute Co., LaGrange, Illinois), diluted with Liquiflor (Pilot Chemicals, Inc., Watertown, Massachussetts) and counted in a Nuclear-Chicago liquid scintillation counter, together with a standard solution of [^{3}H]ABA–tyr.

RESULTS

The same dose (10^{-5} mole) of ABA–tyr that produced unresponsiveness in newborn guinea-pigs (Jones and Leskowitz, 1965a) was administered by intracardiac injections to adult guinea-pigs. At intervals from 1 week before to 2 weeks after this injection, groups of guinea-pigs were immunized with 100 μg ABA–polytyrosine in adjuvant. Two weeks after immunization, their delayed skin reactivity to the hapten was tested with the

TABLE 1

EFFECT OF INJECTION OF ABA–tyr ON DEVELOPMENT OF HAPTEN-SPECIFIC DELAYED SENSITIVITY IN GUINEA-PIGS IMMUNIZED WITH 100 μg ABA–POLYTYROSINE

Group	No.	Treatment	Average delayed reaction* to: ABA–GSA (5 μg N)	Average delayed reaction* to: OT (1:500)
I	5	10^{-5} mole ABA–tyr intracardially at −14 days†	6	12
II	8	Same at − 7 days	7	9
III	8	Same at − 1 day	9	8
IV	8	Same at day 0	5	12
V	8	Same at + 1 day	9	11
VI	8	Same at + 7 days	13	9
VII	5	Controls, no ABA–tyr	13	9

* mm of induration and erythema.
† Day 0 is day of immunization.

azobenzenearsonate conjugate of guinea-pig serum albumin (ABA–GSA). As may be seen in Table 1, an injection of ABA–tyr from 2 weeks before till the day after immunization produced some decrease in the development of hapten-specific delayed sensitivity. An injection 1 week after immunization seemed to be without effect. The specificity of this decrease in sensitivity could be seen from the response to the unrelated old tuberculin antigen (OT). With this antigen, skin reactions in treated animals were occasionally larger but never significantly smaller than reactions in the control animals.

In order to determine whether a greater degree of suppression could be produced with

larger doses of ABA–tyr, a similar experiment was performed in which groups of guinea-pigs received either the same 10^{-5} moles of conjugate or 3×10^{-5} moles given in three divided doses the week before or the week after immunization. This time immunization was performed with the same ABA–tyr, which has been shown (Leskowitz, Jones and Zak, 1966) to produce even more marked hapten-specific delayed sensitivity than the ABA–polytyrosine, since more antigen may be incorporated in the emulsion.

The results in Table 2 demonstrate that as in the previous experiment 10^{-5} moles ABA–tyr given intraperitoneally this time, 1 week before immunization, produces a specific decrease in hapten-directed delayed sensitivity not evident when the same dose

TABLE 2

EFFECT OF INJECTIONS OF DIFFERENT DOSES OF ABA–tyr ON DEVELOPMENT OF HAPTEN-SPECIFIC DELAYED SENSITIVITY IN GUINEA-PIGS IMMUNIZED WITH 10^{-7} moles ABA–tyr

Group	No.	Treatment	Average delayed reactions* to: ABA–GSA (5 μg N)	OT (1:500)
I	5	10^{-5} moles ABA–tyr (i.p. on day −7†)	12	15
II	7	3×10^{-5} moles ABA–tyr (i.p. on days −7, −6, −5)	7 er‡	14
III	5	10^{-5} moles ABA–tyr (i.p. on day +7)	16	15
IV	8	3×10^{-5} moles ABA–tyr (i.p. on days +7, +8, +9)	14	14
V	8	10^{-5} moles ABA–tyr (i.m. and i.p. on day of skin test (+14))	10 er §	N.D.
VI	4	Controls, no injections	19	15

* mm of induration and erythema.
† Day 0 is day of immunization.
‡ Erythema alone, no induration.
§ 4 days later repeat skin test was 17 mm induration and erythema.

is given 1 week after immunization. When three times this dose is divided over the 7th, 6th and 5th days prior to immunization, an almost total suppression of hapten-specific delayed sensitivity results. The same dose given over the 7th, 8th and 9th days after immunization has only a slight effect. Once again, the hapten-specificity of this unresponsiveness is seen in the reactions to OT produced by all groups which were unaffected by the treatment with ABA–tyr.

Immunization of guinea-pigs with ABA–tyr leads to hapten-specific delayed sensitivity with little or no detectable antibody formation. In an effort to determine the effect of prior injections of ABA–tyr on the development of antibody directed to the ABA group, a similar experiment was performed in which guinea-pigs were immunized with a conjugate made from guinea-pig γ-globulin (ABA–GGG). This procedure has been shown to lead to both hapten-specific delayed sensitivity and antibody formation (Jones and Leskowitz, 1965b).

In the first experiment attempted (Table 3), prior injection of 3×10^{-5} moles of ABA–tyr produced an almost total suppression of the hapten-specific delayed sensitivity following

immunization with ABA–GGG. Injection of the same material 1 week after immunization resulted in some decrease in sensitivity to the ABA group but not nearly to the extent shown by the first group. The specificity of this suppression was again shown by the unaffected reactivity to OT in all groups.

TABLE 3

EFFECT OF INJECTION OF ABA–tyr ON DEVELOPMENT OF HAPTEN-SPECIFIC DELAYED SENSITIVITY IN GUINEA-PIGS IMMUNIZED WITH 100 μg ABA–GGG

Group	No.	Treatment	Average delayed reaction* to: ABA–BSA (20 μg)	OT (1:500)
I	5	3×10^{-5} moles ABA–tyr intracardially at −7 days	sl. er.	14
II	7	3×10^{-5} moles ABA–tyr intracardially at +7 days	8	15
III	5	Controls, no injection	13	15

* mm of duration and erythema.
† Day 0 is day of immunization.

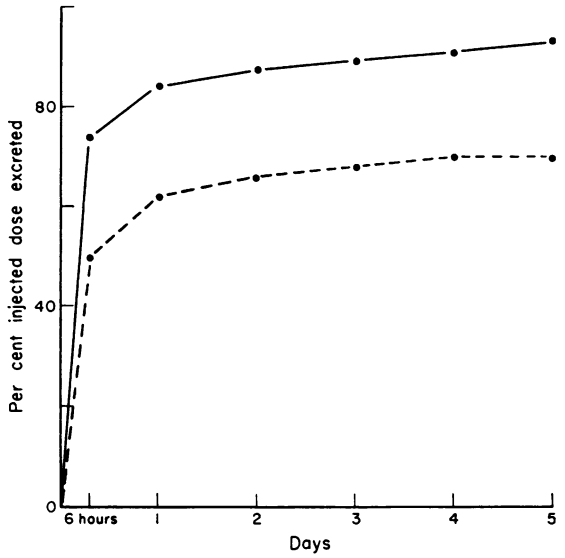

FIG. 1 Excretion of tritium-labelled *N*-acetyltyrosineazobenzene arsonate following intracardiac (—) and intraperitoneal (- - -) injection in guinea-pigs (average, two guinea-pigs).

Little information could be gathered concerning the effect of such treatment on antibody formation, however, since in this particular experiment only low titres were detected by *in vitro* assay with haemagglutination of tanned red cells. However, the experiment did demonstrate an absence of carrier effect in that prior administration of ABA–tyr was able to inhibit the development of hapten-specific delayed sensitivity, whether immunization was attempted with ABA–tyr, ABA–polytyr or ABA–GGG.

The persistence of the low molecular weight conjugate ABA–tyr was studied by following the excretion of tritium-labelled material from normal adult guinea-pigs. The results in Fig. 1 show that within 6 hours following intracardiac injection of approximately 10^{-5}

mole of ABA–tyr almost 80 per cent is excreted in the urine. By day 5, over 95 per cent of the material is excreted. Following autopsy, highest residual activity was found in the liver and the kidneys. Excretion following intraperitoneal injection was not quite as rapid and appeared to level off at 70 per cent after 2 days. The residual activity, however, could not be accounted for in the liver, kidneys, lungs, spleen or blood. This failure to recover all the radioactivity injected could be attributable to trapping in the peritoneum, leakage of material out along the injection track or failure to deliver the full dose because of inability to rinse out the syringe.

Despite the fact that injection of ABA–tyr, even in complete Freund's adjuvant, does not lead to antibody production within the period studied, it was of interest to see whether the presence of antibody specific for the ABA group would have an effect on the development of delayed hypersensitivity to the ABA group. Accordingly, guinea-pigs were treated

TABLE 4

EFFECT OF RABBIT AND GUINEA-PIG ANTI-ABA ANTIBODY ON DEVELOPMENT OF HAPTEN-SPECIFIC DELAYED SENSITIVITY IN GUINEA-PIGS IMMUNIZED WITH 100 μg ABA–POLYTYROSINE

Group	No.	Treatment	Average delayed reaction* to: ABA–GSA (5 μg N)	OT (1:1000)
I	6	0·5 ml rabbit antiserum i.p. on days −1, 0, +1†	16	15
II	6	0·5 ml normal rabbit serum i.p. on days −1, 0, +1	17	14
III	6	0·5 ml rabbit antiserum i.p. on days +7, +8, +9	20‡	14
IV	6	0·5 ml normal rabbit serum i.p. on days +7, +8, +9	19	15
V	6	2·0 ml guinea-pig antiserum i.p. on days 0, +1, 2	20	14
VI	12	Controls, no injections	17	14

* mm of induration and erythema.
† Day 0 is day of the immunization.
‡ All animals had moderate Arthus reactions at 3 hours.

at the time of immunization with anti-ABA serum prepared in rabbits and guinea-pigs. The serum prepared in rabbits gave a precipitin band in agar when set up with ABA–BSA. The guinea-pig antiserum did not give a precipitin band in agar but had a PCA titre of at least 1:20. Administration of these sera at the time of immunization or 1 week later (Table 4) had no effect on the subsequent development of hapten-specific delayed hypersensitivity. In one case (Group III), moderate Arthus reactions were observed at 3 hours, but these did not significantly alter the subsequent development of the delayed reactions.

DISCUSSION

In addition to a further demonstration of the hapten-specific nature of the response to arsanilic acid conjugates, the findings reported here that the injection of ABA-conjugates prior to immunization with adjuvant profoundly depresses the resulting hapten-specific delayed sensitivity, confirm and extend the observations of others using protein antigens (Asherson and Stone, 1965; Dvorak *et al.*, 1965) or contact sensitizers (Sulzberger, 1929;

Chase, 1946). Asherson and Stone (1965) have suggested the name 'immune deviation' for this phenomenon, which in their hands was found to result in a suppression of delayed sensitivity with only slight change in circulating antibody. They felt, moreover, that this process was distinct from 'paralysis', which was a loss of antibody production as well and could be achieved only by high doses of antigen. Dvorak *et al.* (1965), however, found that the unresponsiveness achieved in guinea-pigs by prior injection of comparable doses of antigen extended to immunoglobulin production as well. The degree and type of unresponsiveness produced were dependent on the dose of 'paralysing' antigen given and on the amount of antigen used for immunization. While the results presented in this paper also relate only to the suppression of delayed hypersensitivity, this is largely a consequence of the system chosen, since immunization with ABA-conjugates leads under ordinary circumstances only to delayed sensitivity, with little or no demonstrable antibody production. The findings of Dvorak *et al.* (1965) certainly suggest that this suppression is a general phenomenon affecting all the separate components of the immune response.

The relation of this phenomenon to classic immunological tolerance is of considerable interest and merits further discussion. Although there are indications to the contrary (Humphrey and Turk, 1961), the difficulty in producing immunological tolerance in neonatal guinea-pigs has been frequently attributed to the immunological maturity of the newborns of this species in contrast to mice, rats and rabbits. Therefore, the apparent ease of production of unresponsiveness in the adult guinea-pig by injection of antigen prior to immunization with adjuvant might be taken to represent an entirely different phenomenon unrelated to classic tolerance.

It is possible, however, to present a unifying hypothesis to reconcile these different observations. First, it has been observed that single injections of many protein antigens produce no apparent immune response in adult guinea-pigs (Weigle and Dixon, 1957). It has also been shown for the production of tolerance in adult mice to at least one antigen that a single injection will produce no immune response (Dresser, 1962). Similarly, injections of monovalent conjugates without adjuvants into adult guinea-pigs do not induce a detectable immune response. Thus, we might envisage a number of situations where precursor cells may be exposed to antigen without an ensuing immune response. This could either be attributable to host factors (e.g. immunological immaturity) or antigen factors (e.g. lack of 'adjuvanticity', Dresser, 1962). The net result of such contact would be paralysis of the cell with inability to react when proper conditions for an immune response are present.

It is of interest in this connection that most of the reported instances of unresponsiveness in respect to antibody production in adult animals involved subsequent immunization with adjuvant. In addition, similar unresponsiveness to delayed hypersensitivity has been reported in rats and rabbits (Paterson, 1959; Waksman, 1959). The possible exceptions involve contact-type sensitivity, where unresponsiveness has been shown following percutaneous immunization (Sulzberger, 1929; Chase, 1946), as well as adjuvant immunization (Lowney, 1965).

The failure of passively administered antibody to suppress the hapten-specific delayed sensitivity (Table 4) is also consistent with this concept that the initial 'paralysing' effect of antigen is not produced through an antibody response.

The necessity for an adjuvant to convert a non-immunizing stimulus to an immunizing one may be looked at as a process of either directing the antigen to an appropriate cell, putting the antigen in an appropriate form, or producing a new population of responding

cells. The failure of adjuvant to break tolerance in the systems under consideration argues against the last possibility and suggests that the way in which an antigen reaches the responding cell determines whether it is suppressed or stimulated (Mitchison, 1964; Dresser, 1965). One attractive possibility, deserving further investigations, is that antigen directly impinging on the precursor cells produces tolerance, while that coming by way of the macrophage system produces immunity (Nossal and Austin, 1966).

The importance of the timing of antigen injection relative to the immunization with adjuvant is confirmed in the present studies. Thus, conjugates given as much as 2 weeks before, to at least the day after, immunization have a similar suppressive effect. The degree of suppression produced seems clearly related to the dose administered. However, the related questions of the persistence of conjugate and the site of its action are more difficult to ascertain. Because of the extraordinary rapidity with which this small conjugate is cleared following intravenous injection (Fig. 1), it seems unlikely that circulating material present at the time of immunization is responsible for the production of unresponsiveness. The degree of unresponsiveness produced by 10^{-5} moles given 2 weeks previously is the same as that given at day 0 (Table 1), even though the concentration in the circulation would have been considerably smaller by the time of immunization in the first case.

It is difficult from the data presented here to determine whether sequestered material is involved in the production of unresponsiveness. Thus, while it was seen that considerably more conjugate is excreted in a week following intravenous injection (95 per cent) than intraperitoneal injection (70 per cent), the degree of unresponsiveness produced by equal amounts given either way was comparable. In our previous studies on production of tolerance with this system in neonatal guinea-pigs (Jones and Leskowitz, 1965a), the conjugates were administered by intradermal, intramuscular and intraperitoneal routes. While no quantitative estimates of the persistence of conjugate given this way could be made, it seems most likely that it would be greater than for material given by either intravenous or intraperitoneal routes alone. There is no operational way in this system to distinguish a cell that becomes paralysed prior to immunization from one that becomes paralysed during immunization. It is, therefore, possible that the amount of conjugate present at immunization at 6 weeks, but not at 8 weeks, was sufficient to produce the unresponsiveness seen but far more likely that the critical factor is the initial concentration relative to the number of potentially competent cells and that breakthrough or loss or tolerance is achieved via a turnover in cell population (Mitchison, 1961).

There was a clear cut difference in the effect of conjugates administered prior to immunization in comparison with that given after sensitization has occurred. One week after immunization, administration of conjugates has only a slight suppressive effect on the development of delayed sensitivity (Tables 2 and 3). Conjugates given 2 weeks after immunization (day of skin test) again totally suppresses the response (Table 2), but as shown previously (Leskowitz and Jones, 1965), this suppression is only transient and sensitivity returns within a few days. It seems clear, therefore, that the transient suppression of an already existing sensitivity requires a high concentration of circulating conjugate at the time of skin test, involving reversible interaction, but presumably has no lasting effect. It is possible to argue from these data that the cells affected by conjugate given prior to immunization are qualitatively different from those affected transiently after sensitization is achieved. Thus, we could envision a situation where tolerance could be achieved by appropriate amounts of antigen at a critical time in a cell's life (rather than in an animal's life) and that once a cell embarked on a pathway leading to an immune response, the

process was irreversible for that cell. This might account for the relative difficulty of producing tolerance in an already immunized animal (Mitchison, 1961; Dresser, 1965), where it has been suggested that the acquired tolerance is noticeable only with the terminal exhaustion of the immunologically differentiated antibody producing cells (Sterzl, 1966).

It is recognized that the data on suppression presented here involve only delayed hypersensitivity and that the discussion has considered an antibody immune response and a cellular type sensitivity response interchangeably. The conceptual basis for this (which remains to be proven by future extensions of these studies) is that all the possible immunological responses represent separate but parallel pathways. Each pathway may be initiated or inhibited independently by any given antigen, depending on such factors as dose, type of carrier, presence of adjuvants, etc. Each pathway has a similar mechanism for identifying an antigenic determinant (a light and a heavy chain?), but each pathway results in a final effector substance with a different biological effect (IgG, IgM, skin sensitizing antibody, delayed sensitive cell).

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