TRANSFER OF DELAYED HYPERSENSITIVITY TO DIPHTHERIA TOXIN IN MAN

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Hypersensitivity reactions of the delayed inflammatory tuberculin type are considered to be initiated by an immunological process because they occur only in the specifically sensitized host. Nevertheless, no clear cut relationship between this type of hypersensitivity and conventional serum antibody has been demonstrated, although frequently such circulating antibody is present with its specificity directed against the same substance that can elicit the delayed hypersensitivity reaction. Unlike hypersensitivity of the immediate type, serum from donors showing the delayed type of hypersensitivity does not ordinarily confer sensitivity when injected into non-sensitive recipients.

Transfer to normal guinea pigs of delayed sensitivity to simple chemicals and to tuberculin, by means of living leucocytes was first demonstrated using peritoneal exudates from sensitized guinea pigs (Landsteiner and Chase (1); Chase (2)). In man, transfer of delayed sensitivity to tuberculin and to certain streptococcal proteins is readily accomplished by subcutaneous or intradermal injections of white cells from the peripheral blood of sensitive donors into non-sensitive recipients (3, 4). The leucocyte factor involved in transfer may be extracted from the cells by disrupting them by repeated freezing and thawing, and is not destroyed by desoxyribonuclease, ribonuclease (5), or by treatment with crystalline trypsin (6).

The nature of the factor or factors responsible for transfer of delayed hypersensitivity in man and their relation to serum antibody are not known. This may be due, in part at least, to the fact that the tuberculin and streptococcal products that have been used as test materials are neither homogeneous nor well defined and that no sensitive method is available for determination of small amounts of specific antibody, even if present.

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Some years ago we showed that the delayed inflammatory reactions often observed in adults following intradermal injection of materials used in the Schick test could be provoked by minute doses of highly purified diphtheria toxin or toxoid (7). In a large series of sensitive individuals studied, detectable circulating antitoxin was always present, although no definite correlation between antitoxin titer and degree of sensitivity could be established. The diphtheria system would appear well suited for study of the relationship of circulating antibody to hypersensitivity of the delayed type, since antitoxin may be determined quantitatively even when present in very low concentration (<0.01 μg. per ml.).

In the present paper we report on the successful transfer of sensitivity to purified diphtheria toxin or toxoid[1] in eight successive Schick-positive nonsensitive individuals, using extracts from white blood cells taken from sensitive donors. Specific induction of delayed hypersensitivity to toxin in Schick-positive individuals by minute amounts of toxoid injected intradermally in the form of a toxoid-antitoxin complex (8) is also reported.

Materials and Methods

Schick Test Materials.—Purified diphtheria toxin and toxoid were supplied to us through the courtesy of Dr. James A. McComb, Biologic Laboratories, Massachusetts Department of Health, Jamaica Plain. The test dose of toxin contained 0.0011 L_f units and 0.00083 μg. protein nitrogen (purity about 60 per cent). The toxoid control dose contained 0.009 L_f and 0.0046 μg. protein nitrogen (purity about 90 per cent).

Purified Toxoid.—Preparation KP28 containing 50 L_f/ml. toxoid was used for immunization and for preparing the reagents used to demonstrate the specificity of the delayed reactions in sensitive individuals. This toxoid had been shown to be 95 per cent specifically precipitable by horse antitoxin in three laboratories (9).

Tuberculin.—Old tuberculin (O.T.) 1:1000 prepared by the New York City Department of Health was used for the selection of tuberculin-negative recipients. PPD tablets (Sharp and Dohme, Philadelphia) containing no preservative were diluted in sterile saline to the desired strength.

Antitoxin Titrations.—The intracutaneous method of titration in rabbits according to Fraser (10) was employed. The standard National Institute of Health antitoxin was used for comparison. No attempt was made to determine antitoxin titers less than 0.001 units per ml. (0.0025 μg. antitoxin nitrogen per ml.).

Human Antitoxic γ-globulin.—Serum was drawn from volunteers 12 to 14 days following a "booster" injection of 50 L_f purified diphtheria toxoid. 6.75 ml. pooled serum, containing 110 units/ml. antitoxin as determined by quantitative precipitation (11) and 140 units/ml. by rabbit skin test, were fractionated with alcohol at low temperature according to the method of Lever *et al.* (12). The precipitated γ-globulin fraction was made up to 10 ml. and sterilized by filtration. It contained 12 mg. protein per ml. and 80 units/ml. antitoxin by rabbit skin test. A quantitative precipitin reaction with purified diphtheria toxin was carried out and gave a curve characteristic of a single antigen-antibody system. The solution

[1] Toxin and toxoid are equally effective and indistinguishable from one another in provoking delayed reactions. Therefore, reactions elicited with toxoid indicate sensitivity to toxin.

contained 900 μg./ml. specifically precipitable antitoxin equivalent to 60 *in vitro* units per ml. (11).

Gamma globulin was similarly prepared by alcohol fractionation of 5 ml. pooled serum from Schick-positive individuals. The pool contained <0.001 units/ml. antitoxin.

Antitoxin-Absorbed Toxoid.—To 1 ml. antitoxic γ-globulin (60 *in vitro* units) were added 1.6 ml. saline and 0.4 ml. toxoid KP28 (50 L_f/ml.). The mixture was incubated for 30 minutes at 37°C. and then allowed to stand overnight in the cold. The specific precipitate was removed by centrifugation and the supernate collected and diluted 1:670 in buffered saline containing 0.25 per cent human serum albumin.

0.2 ml. (10 L_f) toxoid KP28 was added to 0.8 ml. gamma globulin prepared from Schick-positive serum. No precipitation occurred. The mixture was diluted 1:100 in buffered serum albumin to contain 0.1 L_f toxoid per ml. equivalent in strength to the standard Schick control solution. All operations were carried out under precautions for sterility.

The above solutions were used to establish the specificity of delayed skin reactions to toxoid. The specific toxoid-antitoxin precipitate was washed twice with 2 ml. chilled saline and then suspended in 2 ml. saline (10 L_f/ml. complex). This suspension of specific precipitate was used to induce sensitivity to toxoid in Schick-positive subjects as outlined below.

Preparation of Leucocyte Extracts for Transfer.—Leucocytes were isolated from donor's heparinized blood (0.1 mg. crystalline heparin, Connaught, per ml. of blood). Bovine fibrinogen (fraction I, Armour) was added and the leucocyte layer was collected as described previously (5).

The packed white blood cells were usually resuspended and washed twice in 10 ml. changes of the recipient's plasma and finally suspended in a small volume of recipient plasma. Unless otherwise indicated, the cells were then disrupted by 7 to 10 cycles of freezing and thawing and treated with crystalline pancreatic desoxyribonuclease (Worthington) in the presence of added Mg^{++}. This extract was injected intradermally and subcutaneously in several sites over the deltoid area of the non-sensitive recipient. All skin tests were performed on the forearms.

EXPERIMENTAL

Selection of Donors.—Three Schick-negative individuals showing pronounced delayed inflammatory reactions of the tuberculin type to purified diphtheria toxoid were selected as donors from among a group of about 250 medical students who were Schick-tested. All three donors were also found to be moderately sensitive to old tuberculin.

That the donors were sensitive to diphtheria toxoid itself, is shown by the fact that in each case delayed skin reactions were markedly reduced in intensity after specific removal of the toxoid component by precipitation with excess human antitoxic γ-globulin. Table I shows the dimensions of skin reactions in each donor 48 hours after intradermal injection of 0.01 L_f purified toxoid in diluted γ-globulin from Schick-positive individuals and of the supernate from an equivalent amount of toxoid after its specific removal by antitoxic γ-globulin as outlined under Materials and Methods.

The reactions to the toxoid-free supernate were probably elicited by traces of non-toxoid diphtherial protein (*ca.* 5 per cent) present as impurity in the toxoid preparation. As shown previously (7) most individuals who are sensitive to toxoid also show delayed sensitivity to other diphtherial proteins.

Selection and Sensitization of Recipients.—The recipients were chosen from the same group of students and all showed strongly positive reactions to toxin when examined on the 3rd or 4th day following the Schick test. In no instance were reactions of any kind to the control toxoid observed and all recipients showed less than 0.001 unit antitoxin per ml. serum at the time of cell transfer. In three of the recipients studied, the Schick test had been performed more than 15 months prior to cell transfer. Only two of the eight (*Yo* and *Gu*) showed positive reactions to 1:1000 old tuberculin.

1. Yo.—3 days after the Schick test, *Yo* was sensitized with the DNase-treated extract from 0.6 ml. of packed, washed leucocytes obtained from donor *Gr*. Skin tests with Schick

TABLE I

Specificity of Donors' Reactions to Purified Diphtheria Toxoid

Donor	Serum antitoxin titer	Skin Reactions at 48 hrs*		
		0.01 L_f toxoid in Schick (+) gamma globulin	Supernate‡ after absorption with antitoxic globulin	Diluent
	units/ml	*mm.*	*mm.*	*mm.*
Gr	0.5	91 × 50 (12 × 11)	31 × 28 fading (7 × 6)	8 × 8 (5 × 4)
Wo	2	80 × 55 (12 × 12)	28 × 20 (16 × 10)	No reaction
Th	>1<10	55 × 30 (15 × 15)	20 × 18 (5 × 5)	No reaction

* Figures in parentheses denote dimensions of central area.

‡ After specific precipitation of toxoid by excess antitoxic globulin and dilution (see Materials and Methods).

control toxoid were carried out 1, 9, and 20 days after transfer. A total of 0.036 L_f (0.11 μg.) toxoid was injected. Maximal sensitivity was observed on the 9th day when the skin reaction at 24 hours measured 25 × 25 mm. with induration and tenderness. The subject was still Schick-positive when tested 31 days post transfer.

2. St.—The cell transfer was given 3 days after Schick test with DNase-treated extract from 0.6 ml. of washed leucocytes from donor *Gr*. Prior to freezing and thawing, the cells were suspended in 1 ml. *St* plasma. Total antitoxin of disrupted cell extract was less than 0.01 unit as determined by rabbit skin test. Skin tests with Schick control toxoid were carried out 1, 9, and 20 days after transfer. The total toxoid injected was 0.036 L_f (0.11 μg. toxoid protein). Maximal sensitivity was observed on the 9th day and the delayed skin reaction to toxoid measured 25 × 35 mm. with 10 × 10 central area at 24 hours. At 20 days a negative reaction was obtained to 1/500 M.L.D. toxin ($\frac{1}{10}$ usual Schick test dose) and a serum level of 0.002 units/ml. of antitoxin was found at this time. 11 days later the serum contained more than 0.01 unit antitoxin per ml.

3. Ro.—The cell transfer was given 7 days after the Schick test with DNase-treated extract from 0.4 ml. washed leucocytes from donor *Wo*. Cells were suspended in *Ro* plasma

before freezing and thawing. 2.4 ml. of extract were injected, containing <0.01 unit antitoxin per ml. 4 hours after transfer, a flare-up occurred at the site where Schick toxin had been injected 7 days previously. This reaction measured 45 × 25 mm. with 20 × 20 central indurated area, and persisted for more than 48 hours. Skin tests with Schick control toxoid were performed at 5 and 16 days. Maximal sensitivity occurred on the 5th day after transfer and 24 hours later the skin reaction to toxoid measured 15 × 15 mm. At the same time Schick toxin caused a more severe inflammatory reaction of the delayed type measuring 45 × 30 mm. and increasing to 35 × 70 mm. with 15 × 25 central area at 72 hours. This allergic reaction then subsided leaving the characteristic Schick-positive pigmented area. *Ro* was still Schick-positive even to $\frac{1}{10}$ the usual Schick test dose of toxin on the 16th day after transfer but on the 28th day post transfer his serum contained 0.01 unit antitoxin per ml.

4. Bl.—Cell transfer was given 7 days after Schick test with DNase-treated extract from 0.4 ml. washed leucocytes from donor *Wo*. Cells were suspended in *Bl* plasma before freezing and thawing. 2.5 ml. of extract was injected containing <0.01 unit antitoxin per ml. On the 5th day after transfer, skin test with old tuberculin was positive (1+ at 24 hours), indicating that transfer was successful. Skin test with toxoid was withheld until the 19th day. The delayed reaction to toxoid measured 20 × 15 mm. when seen at 24 hours. Serum drawn on the 28th day after transfer still contained less than 0.001 unit antitoxin per ml.

5. Gu.—The Schick test had been performed 27 months previously. 14 days before transfer a serum sample showed less than 0.001 unit antitoxin per ml. 0.6 ml. packed washed leucocytes from donor *Wo* were suspended in 3 ml. freshly drawn *Gu* serum to which 1 L_f purified diphtheria toxoid had been added. After 30 minutes' incubation of the cell suspension at 37°C. with occasional gentle shaking, the suspension was centrifuged and the slightly turbid, but cell-free supernate, was injected intradermally and subcutaneously into *Gu* in the deltoid region. Skin tests with Schick control toxoid performed on the 4th, 11th, 18th, and 49th days after transfer, all caused delayed reactions. Maximal sensitivity was observed 49 days after transfer when the 24 hour reading of the skin test measured 20 × 20 mm. In addition to the initial 1 L_f (3 μg.) toxoid, a further total of 0.045 L_f (0.14 μg. toxoid protein) was injected intradermally. 83 days after transfer the serum antitoxin level remained less than 0.001 units per ml.

6. Ta.—The Schick test was performed 59 days before cell transfer. The cell residue after treatment with toxoid in *Gu* serum (Case 5) was washed 2 times with 10 ml. changes of fresh *Ta* serum and then suspended in 2 ml. *Ta* serum. The cell suspension was frozen and thawed, and treated with DNase in the usual manner. The final volume of extract injected was 3.7 ml. Skin tests with Schick control toxoid were performed on the 4th, 11th, 18th, and 28th day post transfer. Sensitivity increased to a maximum on the 11th day when the 24 hour reading of the skin test measured 15 × 15 mm. Sensitivity declined thereafter and the skin test measured only 2 × 2 mm. on the 28th day.

7. Ho.—1.42 ml. of packed cells from donor *Th* were washed twice with 2 per cent human serum albumin (HSA) in saline. They were resuspended in 3 ml. HSA-saline at 37°C. for 1 hour and then centrifuged. The cell-free supernate (2.6 ml.) was injected intradermally and subcutaneously into *Ho*. 4 days later skin test showed a 10 × 10 reaction to Schick toxoid which was rated 1+ when seen at 24 hours. The centrifuged cells were divided into equal portions. 0.7 ml. was resuspended in 1 ml. *Ho* serum + 1 ml. HSA-saline containing 0.1 L_f toxoid and 0.1 mg. $MG{\cdot}SO_4{\cdot}7H_2O$ and incubated at 37°C. for 1 hour. The cells were then removed by centrifugation and the supernate stored in deep freeze for 5 days. It was then thawed and injected into *Ho*. 6 days later the reaction to Schick control toxoid measured 20 × 35 mm. with induration and tenderness when seen at 24 hours and was rated 2+. The subject was still Schick-positive 63 days after transfer.

8. Me.—The remaining aliquot of 0.7 ml. washed *Th* cells (see Case 7) was suspended in

Me serum containing toxoid and Mg^{++}. After 1 hour at 37°C. the cell-free supernate was injected into the skin over the deltoid region of *Me*. Maximal sensitivity was observed on the 4th day when the toxoid control reaction at 24 hours measured 12 × 12 mm. and was rated 1+. At 21 days sensitivity was diminishing and a reaction measuring only 6 × 5 mm., rated ±, was observed at 24 hours. The subject was still Schick-positive 69 days after transfer.

Results of Transfer Experiments.—Table II summarizes the transfers from three donors sensitive to diphtheria toxin (or toxoid) and to tuberculin. It will be seen that all eight Schick-positive non-sensitive recipients[2] responded to transfer and became hypersensitive both to purified diphtheria toxin and to old tuberculin. The fact that the degree of sensitivity developed to toxoid and to tuberculin was not as great as that often attained in previous studies (5) can be attributed to the fact that the donors used in the present series were not as exquisitely sensitive as those used previously.

The present study indicates that delayed sensitivity develops promptly following transfer. All the recipients who were skin-tested within 24 hours after receiving leucocyte extracts showed unequivocal delayed reactions to toxoid rated 1+ or better. The degree of hypersensitivity tended to decline somewhat 2 to 3 weeks after transfer.

Only two recipients, *St* and *Ro*, developed detectable circulating antitoxin following transfer and the subsequent skin tests with toxoid. In both instances, however, no antitoxin (*i.e.*, <0.001 unit per ml.) could be detected in serum drawn at the time of maximal sensitivity. On the other hand, the fact that these two individuals did develop significant serum antitoxin levels 3 and 4 weeks after transfer suggests that a very low basic antitoxic immunity may have existed at the time of transfer, and that skin test with toxoid caused a secondary response (13). None of the other recipients developed detectable antitoxin despite repeated skin tests. This is not surprising, perhaps, since the total amount of toxoid injected was small in most cases, varying between 0.06 and 0.16 μg. toxoid protein. A usual course of fluid toxoid for primary immunization consists of three doses totalling 400 to 500 μg. of toxoid protein.

Table II also shows that Schick testing of recipients just prior to transfer has no significant effect on subsequent development of hypersensitivity, since transfer was equally effective in individuals whose Schick tests had been performed 15 to 27 months previously.

Release of Transfer Factor from Leucocyte Suspensions.—The initial series of transfers of hypersensitivity to toxin were carried out using cells which had

[2] Two additional recipients were successfully sensitized to toxin and tuberculin using extracts from *Gr* and *Th* cells which had been stored for 5 and 3 months respectively in the frozen state. This observation furnishes additional evidence for the stability of the transfer factor.

TABLE II

Transfer of Delayed Sensitivity to Diphtheria Toxoid and to Tuberculin with Leucocyte Extracts

Toxoid-sensitive donor	Recipient*, ‡	Recipient Schick test days before transfer	Delayed reaction to 0.009 L_f toxoid		Recipient antitoxin titer	Sensitivity to tuberculin (O.T. 1:1000) 24 hr. reading
			Time after transfer	Severity of Skin reaction at 24 hrs§		
			days		*units/ml.*	
Gr	Yo‖	−3	1	1+	<0.001	
			9	3+	<0.001	————
			20	1+	<0.001	
	St	−3	1	1+	<0.001	Not tested
			9	3+	ca 0.001	1+
			20	1+	0.002	±
			31	Not tested	>0.01	±
Wo	Ro	−7	5	1+	<0.001	1+
			16	±	<0.001	1+
			28	Not tested	0.01	1–2+
	Bl	−7	5	Not tested	—	1+
			19	1–2+	<0.001	Not tested
			28	1+	<0.001	1+
	Gu‖·¶	−730	4	1+	<0.001	
			11	1–2+	<0.001	
			18	1–2+	<0.001	————
			49	2+	<0.001	
	Ta	−59	4	1+	<0.001	±
			11	1–2+	<0.001	1+
			18	1+	<0.001	1+
			28	±	Not done	±
Th	Ho¶	−417	6	2+	<0.001	±
			16	1–2+	<0.001	1+
			63	1+	<0.001	±
	Me¶	−440	4	1+	<0.001	1+
			21	±	<0.001	±

* Final extracts from disrupted washed cells contained <0.01 units antitoxin/ml.

‡ All recipients showed less than 0.001 unit antitoxin/ml. at time of transfer.

‖ Recipients *Yo* and *Gu* were tuberculin-positive at the time of cell transfer.

§ Each 1+ = 10 × 10 mm. erythema and edema.

¶ Sensitized using supernatants from slightly damaged cells. No freezing and thawing. See text.

been disrupted by repeated freezing and thawing and then treated with DNase. This method had already proved successful in the transfer of sensitivity to tuberculin and to streptococcal proteins (5). In the case of three of the transfers summarized in Table II, less drastic methods were used to release the transfer factor and recipients *Gu, Ho,* and *Me* were sensitized with cell-free supernates from cell suspensions which had merely been allowed to remain at 37°C. for short periods of time in the presence of a small amount of toxoid. As an example, let us consider the sensitization of *Gu.* In this case, leucocytes from donor *Wo* after washing twice with recipient's serum, were suspended in 3 ml. fresh *Gu* serum containing 1 μg. purified toxoid per ml. and incubated for 30 minutes at 37°C. The cells were then removed by centrifugation and the slightly turbid supernate injected into *Gu* who when first skin-tested 4 days later with toxoid showed a 15 × 15 mm. inflammatory reaction at 6 hours, diminishing to 10 × 10 mm. when seen at 24 hours. The cell residue obtained above, was washed twice with recipient *Ta* serum and then the cells were disrupted in the usual manner by freezing and thawing. The DNase-treated extract was used to sensitize *Ta.* As seen from Table II, the degree of sensitivity developed by *Ta,* who received the completely disrupted cell residue, was no greater than that developed by *Gu* who received only the cell-free supernate. Recipients *Ho* and *Me* were also sensitized with similarly prepared cell-free supernates from suspensions of donor *Th* cells incubated at 37°C. *Ho* developed definite sensitivity following injection of the supernate from a cell suspension which stood at 37°C. for 1 hour and to which no toxoid had been added.

Tuberculin sensitivity was likewise transferred to two tuberculin-negative recipients[3] with similarly prepared supernates from tuberculin-sensitive cells. Of two equal aliquots of unwashed leucocytes (0.5 ml. packed cells) from an exquisitely sensitive tuberculin positive donor, *Ca,* 1 aliquot was suspended in a solution consisting of 0.5 ml. recipient *Al* fresh serum, 0.5 ml. of 0.5 per cent $MgSO_4 \cdot 7H_2O$ and 25 μg. PPD in 0.5 ml. saline. After standing for 1 hour at 37°C., the cells were removed by centrifugation and the supernate injected into recipient *Al.* The other aliquot of sensitive cells was suspended in 0.5 ml. 2 per cent crystalline human serum albumin in saline. After 1 hour at 37°C. the supernate from the latter was injected into recipient *En.* 3 days after transfer both subjects were skin-tested with 1:1000 O.T. At 24 hours *Al* showed a 30 × 25 mm. area of edema with a 10 × 15 central area and the reaction was rated 3+. At the same time, *En* showed 15 × 15 mm. faint erythema with a 7 × 10 mm. central indurated area and was rated 1+.

The above experiments suggest that release of the transfer factor from sensitive cells may occur under relatively mild circumstances. The experiments also

[3] Recipients *Al* and *En* were tested with tuberculin 48 hours before cell transfer and showed no reaction. Moreover, both had been tested with O.T. 1:1000 one and two years previously with negative results.

demonstrate the failure of specific antigen (toxoid or PPD) to block the transfer. Microscopic examinations of suspensions kept at 37°C. under the above conditions showed that some alterations of the cells had taken place. Dried smears revealed cells containing vacuolated nuclei and the presence of occasional "basket cells." The polymorphonuclear cells appeared to have undergone the greatest morphological change while the lymphocytes appeared to have suffered the least damage. This observation is consistent with the known greater fragility of the polymorphonuclear leucocytes. As previously reported (4), the procedure used to isolate the leucocytes yields preparations in which lymphocytes predominate.

Will Inflammatory Cells Neutralize Diphtheria Toxin in the Sensitized Schick-Positive Recipient?.—Recipients *Ho* and *Me* were sensitized to diphtheria toxin using cell-free supernates prepared from leucocytes obtained from donor *Th.* 4 days after transfer, skin sites on the forearms were injected respectively with 1:100, 1:10, and undiluted Schick control toxoid (*i.e.*, 0.00009, 0.0009, and 0.009 L_f). 6 hours later these sites showed delayed inflammatory lesions rated ±, 1+, and 2+ respectively. Each site was then injected with $\frac{1}{500}$ M.L.D. purified diphtheria toxin ($\frac{1}{10}$ usual Schick test dose). At the same time, fresh sites were injected with a mixture of undiluted toxoid containing $\frac{1}{500}$ M.L.D. toxin and with the toxin alone. The Schick tests were read 4 days later after the allergic reactions had subsided. The inflammatory reactions failed to neutralize the necrotic action of the toxin; indeed the severity of the local damage caused by the toxin was markedly increased in the immediate vicinity of the injection site in direct proportion to the intensity of the allergic inflammation (see Fig. 1). Presumably, the allergic reaction impeded diffusion of toxin away from the site so that the damage was restricted to a smaller area of skin. The apparent enhancing effect of hypersensitivity to toxoid on the subsequent reaction to toxin was equally pronounced when toxin and toxoid were mixed and injected simultaneously into the skin.

Aside from the present series of artificially sensitized subjects, all individuals sensitive to diphtheria toxin that we have tested in the past have shown readily detectable serum antitoxin levels. Clearly the circulating antitoxin in such individuals fails to bind toxin or toxoid injected into the skin sufficiently to prevent initiation of a specific inflammatory response. Nevertheless, it seemed worthwhile to find out whether antitoxin combined with toxoid *in vitro* could prevent the delayed reaction. In three separate instances, a toxoid-sensitive donor's own antitoxin (in excess) was mixed with a small amount of Schick control toxoid and the mixture injected into the donor's own skin. At 48 hours inflammatory reactions to the mixture were as large or larger than to toxoid alone.

Active Induction of the Delayed Hypersensitive State by Injection of Specific Precipitates.—The recent experiments of Uhr *et al.* (8) have shown that guinea

pigs develop a high degree of sensitivity of the delayed type to diphtheria toxin and to crystalline egg albumin following intradermal injection of minute amounts of specific precipitates formed in antibody excess. Animals sensitized in this way form no demonstrable circulating antibody to either toxin or egg albumin.

Four adult subjects whose serum contained less than 0.001 units/ml. antitoxin were specifically sensitized to diphtheria toxoid using washed specific precipitates formed by adding purified toxoid KP28 to either rabbit antitoxin or human antitoxic γ-globulin in excess (see section on Methods and Materials). Schick tests were not performed on these subjects prior to sensitization.

TABLE III

Specificity of Delayed Reactions to Diphtheria Toxoid in Human Subjects Sensitized with Specific Precipitates

Subject	Time of reading skin test	Serum antitoxin	Skin reactions to test materials*		
			0.01 L_f toxoid in Schick + gamma globulin	Supernate after absorption with antitoxic globulin	Schick test‡ allergic reaction
	hrs.	*units/ml.*	*mm.*	*mm.*	
Bo	17	>0.01	10 × 7	0	±
Ke	24	>0.01	15 × 10	0	10 × 8
Mu	40	>0.001 <0.01	60 × 30 (12 × 12)	35 × 32 (12 × 12)	25 × 25
Zi	40	<0.001	80 × 45 (10 × 10)	30 × 25 (10 × 10)	40 × 35 (17 × 15)

* Figures in parentheses denote dimensions of central area.

‡ Reactions in this column were of the delayed inflammatory type. Subsequently, in the cases of *Mu* and *Zi* these sites remained pigmented after the control sites had faded.

Subjects *Bo* and *Mu* received a total of 10 L_f toxoid–rabbit antitoxin complex in ten intradermal sites in the deltoid region of both shoulders. 17 days later both subjects showed delayed skin reactions to 0.01 L_f purified toxoid. When read at 16 hours *Mu* showed a 30 × 18 mm. raised erythematous halo with a 10 × 10 mm. central area. The serum antitoxin level was less than 0.001 units/ml. *Bo*'s reaction measured 8 × 8 mm. and was minimal. His serum antitoxin level was *circa* 0.01 unit/ml. 3 days later both subjects received a booster injection of 0.1 ml. of toxoid–horse antitoxin precipitate containing 2 L_f toxoid intradermally in each shoulder. The injection of the complex caused a severe but localized delayed reaction in *Mu* which persisted for several days. The delayed reactions to tests done 13 days after the booster dose are recorded in Table III. *Mu*'s reaction to the Schick test was still slightly positive at this

time but his serum was now found to contain slightly less than 0.01 units/ml. antitoxin.

Subjects *Zi* and *Ke* received a total of 6 L_f toxoid–human antitoxin suspension intradermally in six sites in the shoulders. Both subjects were skin tested 18 days later and the results are shown in Table III. *Zi*'s serum showed <0.001 unit/ml. antitoxin at this time and his Schick test became positive after the delayed reaction had subsided. *Ke* showed more than 0.01 unit/ml. antitoxin and was Schick-negative. It is of interest that *Ke* gave a history of active immunization 3 to 4 years previously. *Zi* gave a history of diphtheria during infancy 23 years previously. However, both subjects showed <0.001 unit/ml. antitoxin at time of sensitization.

Table III shows that the actively induced sensitivity is highly specific for toxoid. The small reactions shown by *Mu* and *Zi* to the supernate after specific precipitation of the toxoid component could be due to solubility of the toxoid–antitoxin complex, traces of complex of toxoid with incomplete or non-precipitating antitoxin, or possibly to sensitization to small amounts of other diphtherial proteins present as impurity in the toxoid owing to traces of antibody against these proteins in the antitoxin used for preparing the specific precipitates.

DISCUSSION

The relationship between serum antibody and delayed hypersensitivity has never been clear. Failure to resolve this problem has been due in large measure to the fact that the methods employed for inducing the hypersensitive state in animals and man (that is, either by infection or the use of antigen in an adjuvant mixture containing mycobacteria) result in simultaneous production of antibody. Moreover, the methods used for detecting serum antibody have often been less sensitive than the skin reactions used to demonstrate hypersensitivity. We are now reporting eight consecutive successful transfers of delayed hypersensitivity to Schick-positive recipients using leucocyte extracts from donors shown to be specifically sensitive to diphtheria toxin. All eight recipients remained Schick-positive following transfer and their serum contained less than 0.001 units antitoxin per ml. at the time of maximal sensitivity. Four recipients remained Schick-positive and showed no detectable antitoxin for 3 months following transfer despite repeated skin tests with the Schick materials. Failure to show a secondary immune response indicates the absence of latent immunity in these four subjects.

Failure to detect circulating antibody in subjects sensitized by transfer is consistent with the recent findings of others. Thus, Gell and Hinde (14) and Tremaine and Jeter (15) were unable to detect antibody in recipient rabbits sensitized by peritoneal exudate cells from donor animals sensitized to egg albumin by means of mycobacteria-containing adjuvants. Tremaine and Jeter

tested their recipient rabbits by precipitin test and by attempting to passively sensitize guinea pigs to anaphylactic shock using as much as 20 ml. serum per guinea pig. Metaxas and Metaxas-Bühler (16) have reported similar findings following cell transfer using guinea pigs sensitized to egg albumin by mycobacteria-containing adjuvants. Even more convincing have been the recent demonstrations that the delayed type hypersensitive state can be induced in agammaglobulinemic subjects by topical application of dinitrochlorobenzene (Good (17)) (Porter (18)). Finally, Uhr *et al.* (8) have shown that guinea pigs may be rendered highly sensitive to diphtheria toxoid and egg albumin by minute amounts of these proteins injected intradermally in the form of a washed specific precipitate formed with excess homologous antibody. In over 50 animals rendered sensitive to toxin, only one showed as much as 0.001 units antitoxin per ml. of serum. Using this same method we have induced tuberculin-type hypersensitivity in four human subjects, two of whom showed intense delayed skin reactions to 0.025 μg. purified toxoid at a time when no antitoxin could be detected in their serum.

Since the subjects sensitized to diphtheria toxin show no detectable antitoxin, it becomes important to demonstrate the specificity of the delayed reactions elicited by intradermal injection of purified toxoid. All three donors showed markedly reduced skin reactions when injected with purified toxoid from which the toxoid component had been removed by precipitation with a specific antitoxin. The effect of specific removal of the toxoid component is strikingly illustrated in Fig. 2 which shows the skin reactions to these reagents in a Schick-positive subject sensitized to toxoid by the method of Uhr *et al.* (8).

Although occasional reports have appeared claiming that delayed hypersensitivity can be passively transferred to normal animals by means of serum taken from sensitive donor animals, such observations have seldom been confirmed and it is generally agreed that hypersensitivity of the tuberculin type is a cellular phenomenon (19). On the other hand, certain claims of successful transfer of delayed hypersensitivity by means of cell-free exudates or filtrates would appear to be well substantiated. McJunkin (20), for example, injected large doses of living tubercle bacilli into the perionteal cavities of heavily infected guinea pigs and succeeded in sensitizing normal guinea pigs with sterile filtrates obtained from the peritoneal exudates. Crepea and Cooke (21) were able to sensitize 50 per cent of their normal guinea pigs using serum taken from guinea pigs sensitive to poison ivy. The sensitive donor animals were apparently skin-tested with poison ivy extract within 48 hours of collecting the serum. Recently, Cole and Favour (22) have transferred tuberculin hypersensitivity by means of a subfraction of α-globulin isolated from the serum of tuberculous guinea pigs skin-tested within 48 hours of drawing the serum. It will be noted that in all of the successful reports cited, treatment with the specific agent (antigen or haptene) seems to be necessary in order to obtain transfer factor free from cells.

It has been reported many times that tuberculin is toxic for leucocytes taken from sensitive individuals (23–25) although there is by no means agreement in the literature on this point (26–28). Our own experiments suggest that the factor concerned in transfer of delayed sensitivity to toxin or to tuberculin may be released from human leucocytes by allowing them to stand in fresh serum at 37°C. for short periods either with or without specific antigen. These experiments suggest that antigen may promote release of the sensitizing material but cannot be interpreted as a demonstration of a specific cytotoxic effect of either toxoid or PPD on sensitive cells since the possibility of non-specific damage has not been excluded. It has been shown clearly, however, that the transfer factor may be released from sensitive cells in effective amounts under relatively mild conditions and without evidence of extensive morphological damage, at least to lymphocytes.

In conclusion, it would appear that although under conditions such as those encountered in infections, serum antibody may be concerned in specific induction of the hypersensitive state, it is extremely improbable that conventional serum antibody is involved in mediation of the delayed reaction itself. Nevertheless, the hypersensitive individual must possess a substance capable of interacting specifically with antigen upon challenge. It is logical to suppose that this specific substance is located in sensitive cells or at their surface. In the case of cells from individuals sensitive to diphtheria toxin, we must conclude either (1) the antitoxin which the cells contain is present in such small amounts as to escape detection even by the sensitive rabbit intracutaneous test, or (2) that the substance which interacts with toxin does so without neutralizing its toxicity, or (3) that the complex formed with toxin has not been detected because it is highly dissociated under the conditions of testing.

SUMMARY

Simultaneous transfer of delayed hypersensitivity to diphtheria toxin and to tuberculin has been accomplished in eight consecutive instances in man using extracts from washed leucocytes taken from the peripheral blood of tuberculin-positive, Schick-negative donors who were highly sensitive (*i.e.*, pseudoreactors) to purified diphtheria toxin and toxoid. The leucocyte extracts used for transfer contained no detectable antitoxin. The recipient subjects were Schick-positive (<0.001 unit antitoxin per ml. serum) and tuberculin-negative at the time of transfer. All the recipients remained Schick-positive for at least 2 weeks following transfer and in every case their serum contained less than 0.001 units antitoxin at the time when they exhibited maximal skin reactivity to toxoid.

Evidence is presented which indicates that the transfer factor may be released from leucocyte suspensions under mild conditions in which most of the cells appear to remain morphologically intact.

Four adult Schick-positive subjects have been sensitized to diphtheria toxoid by intradermal injection of a few micrograms of purified toxoid in the form of

a washed toxoid-antitoxin precipitate. Two of these sensitized individuals showed severe delayed skin reactions specifically directed against diphtheria toxin (or toxoid) at a time when their serum antitoxin level was less than 0.001 units/ml.

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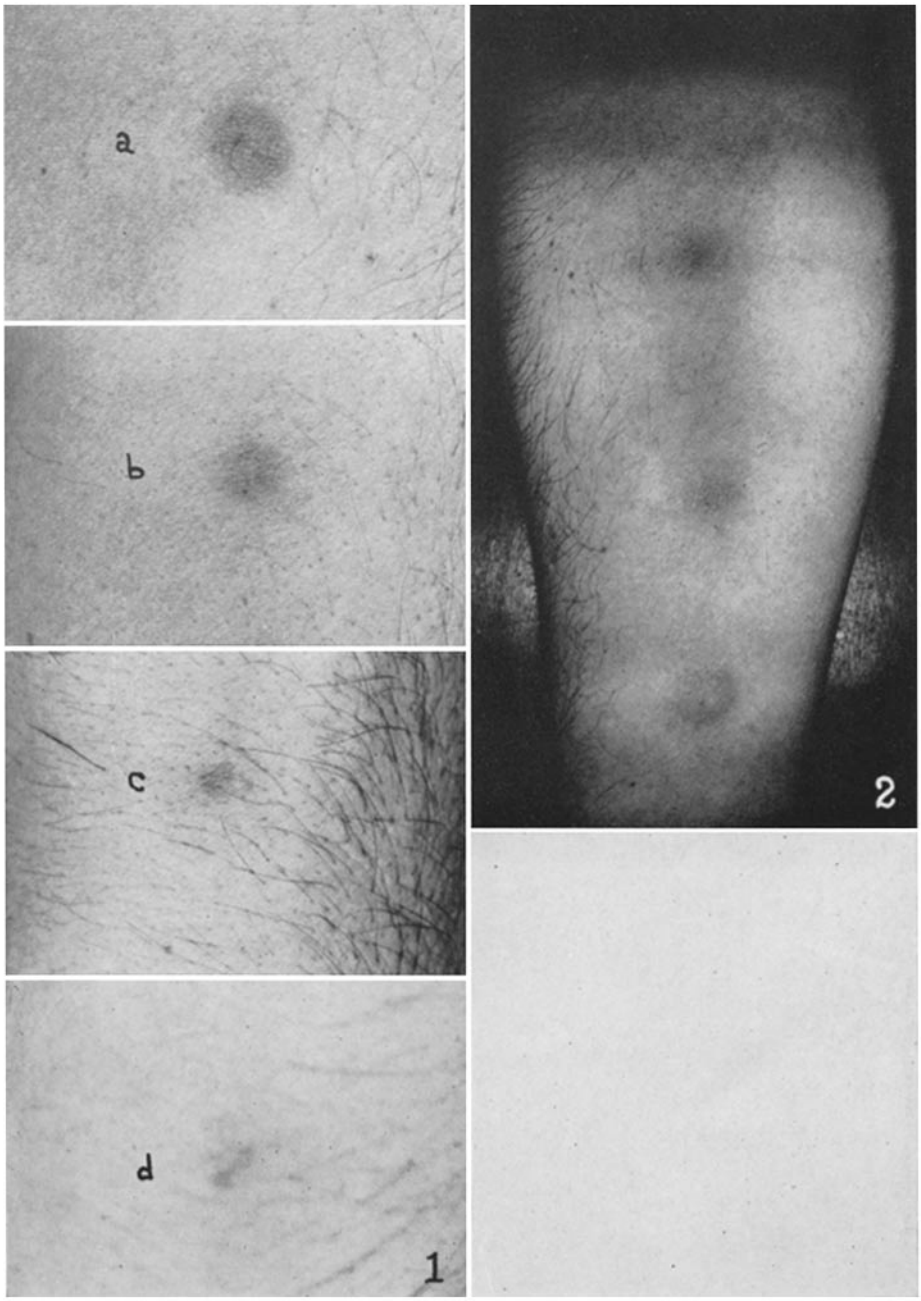
EXPLANATION OF PLATE 27

FIG. 1. Effect of inflammatory reaction to toxoid on dermonecrotic action of toxin. Sites *a*, *b*, and *c* prepared by intradermal injection of 0.009, 0.0009, and 0.00009 L_f purified toxoid respectively. 6 hours later $\frac{1}{500}$ M.L.D. (about 0.0001 L_f) toxin was injected into each site and into control site *d*. Photograph shows appearance of the skin reactions to toxin 4 days later after allergic reactions to toxoid had subsided. See text for further discussion.

FIG. 2. Specificity of induced sensitivity to toxoid. Skin reactions at 24 hours. (Reproduced from a color photograph.) ½ normal size.

Upper, reaction to 0.01 L_f (0.03 μg.) purified toxoid; Middle, supernate from same after removal of toxoid component by specific precipitation with antitoxin; Bottom, Schick test with 0.001 L_f purified toxin. Note blanched central area. This reaction continued to develop after allergic reactions had subsided.

Note that reaction to toxoid (upper) has spread downwards and overlaps reaction to supernate. The extent of upward spread of the toxoid lesion is obscured by a shadow.



(Lawrence and Pappenheimer: Hypersensitivity to diphtheria toxin)