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Transfer of Delayed Hypersensitivity by Lymph-Node Cells in Testis Autosensitization

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Summary. Guinea pigs sensitized by an extract of homologous testis and Freund's adjuvant developed delayed skin hypersensitivity towards a purified testis antigen. When lymph-node cells from sensitized animals were transferred into normal guinea pigs by intravenous, intraperitoneal or intracutaneous injection the recipients also developed delayed skin hypersensitivity.

Maximum reactions in recipients were obtainable after the transfer of cells from donors which had been sensitized for only 6 days. The recipients became sensitized immediately after cell transfer but their sensitization lasted only a few days.

It can be concluded from this and earlier work that sensitized cells as well as free circulating antibody play a part in testis autosensitization.

INTRODUCTION

The testicular atrophy which occurs in guinea pigs after the injection of homologous testis and Freund's adjuvant is widely regarded as an autosensitization reaction (Freund, Lipton and Thompson, 1953, 1955; Voisin and Delaunay, 1955; Waksman, 1959), but it is not known whether the testicular damage is brought about by circulating antibody or sensitized cells.

The serum of sensitized guinea pigs has been shown to contain antibodies which will attach to guinea-pig sperm, as demonstrated by fluorescent staining, and to guinea-pig lung, as demonstrated by subsequent histamine release on addition of antigen (Baum, Boughton, Mongar and Schild, 1961). However, it has not so far been shown that these antibodies can cause testicular atrophy when injected into intact recipients (Freund *et al.*, 1953) although this possibility has not been exhaustively studied.

The skin reactions of sensitized guinea pigs exhibit 'delayed' as well as 'immediate' reaction components which suggests that a hypersensitivity of the tuberculin type, mediated by cell-bound antibody, may also be involved. Freund *et al.* (1953) have pointed out that the need for adjuvant in the production of testicular damage indicates a reaction of the tuberculin type. Nevertheless, the histological investigations of these authors showed that they were primarily degenerative lesions. By contrast, Waksman (1959) concluded from his histological findings that the primary factor in the testicular damage was lymphocytic infiltration and inflammation.

In an earlier investigation (Baum *et al.*, 1961) an attempt was made to obtain more definite evidence on the role of sensitized cells in testis autosensitization by transferring lymph-node cells from sensitized animals into normal recipients and testing for delayed

reactivity to the antigen. The technique of Metaxas and Metaxas-Buehler (1955) was used in which sensitized cells and antigen are mixed together *in vitro* and then injected intracutaneously. These attempts were inconclusive, largely because the sensitized cells themselves produced strong reactions when injected in this way even in the absence of antigen. In the present study we have, therefore, resorted to the older methods of intraperitoneal and intravenous transfer of sensitized cells followed by the intracutaneous administration of antigen.

MATERIALS AND METHODS

ANIMALS. Albino guinea pigs (Pirbright strain) of 450–550 g. weight were used for all experiments.

SENSITIZATION. Antigenic material was prepared from decapsulated guinea-pig testes. The tissue was homogenized with an equal amount (wt./vol.) of saline, strained through muslin to remove fibrous material, and the extract emulsified with an equal volume of Freund's complete adjuvant. Animals were sensitized by intracutaneous injection of a total of 0.4 ml. of this emulsion in multiple sites in the back of the neck.

CELL TRANSFER. Lymph nodes regional to the injection site were dissected out and the cells teased out into Tyrode solution (percentage composition: NaCl 0.8, KCl 0.02, $CaCl_2$ 0.02, $MgCl_26H_2O$ 0.01, $NaHCO_3$ 0.1, $NaH_2PO_42H_2O$ 0.005, glucose 0.1) using two pairs of forceps. After centrifugation the pad of cells was washed by resuspension in Tyrode solution and the number of cells estimated using a Burker counting chamber. The cells were washed a second time, and then resuspended in a convenient volume for injection. In the case of intraperitoneal and intravenous transfers 1–3 ml. were injected containing about 600–700 million cells per ml. The total volume transferred by the intracutaneous route was 0.1 ml. and this contained 5 or 10 million cells alone or with 0.5 mg. antigen.

ANTIGEN. This was prepared from guinea-pig testes in a similar manner to the A.S.P.M. fraction of Freund *et al.* (1955). The yield of dry purified extract corresponded to 0.6 per cent of the wet weight of testes. Ten mg./ml. solution of the dry material in saline was heated to 60° for 30 minutes to destroy hyaluronidase activity, the insoluble precipitate which formed was spun off, and the supernate used for injection. The antigen was injected into the shaved flanks of the guinea pigs. In the case of intracutaneous transfer it was injected with the cells.

MEASUREMENT OF SKIN REACTIONS. The reactions observed 24 hours after antigen injection were mainly flat or only slightly indurated. They were usually oval and sometimes almost rectangular. The product of the two diameters measured in millimetres gave approximately the same result (within 5 per cent) as the measurement obtained by tracing the reaction area on to graph paper. The two main diameters are given in the tables and the areas plotted in the figures.

RESULTS

INTRAPERITONEAL TRANSFER OF CELLS

In one series of experiments the lymph-node cells of testis-sensitized guinea pigs were transferred by intraperitoneal injection into normal guinea pigs. Both donors and recipients

were females except in the first experiment in which male donors were used. The donors had been sensitized for 3 to 7 weeks. From each of these 200–300 million cells were obtained and the cells from several donors were pooled before transferring into recipients. The latter were challenged 48 hours after the cell transfer by intracutaneous injections of 0.05 to 1 mg. purified testis antigen, the reactions being read 24 hours later. The antigen produced no reactions in normal control guinea pigs.

In the first experiment two guinea pigs received 520 million cells each, followed by intracutaneous injections of three doses of antigen (0.05, 0.25 and 1 mg.) and one dose of saline. One of the two recipients failed to give any reaction, the other gave a weakly positive reaction but only with the highest dose of antigen.

In the second experiment, a larger number of cells was used (1940 million) all of which were transferred into a single recipient challenged with two doses of antigen (0.05 and 0.5 mg.) on each flank, and one control dose of saline. All the responses to antigen were positive and the effect was graded according to dose. The reactions consisted of flat or slightly indurated, strawberry-red patches with a clearly defined contour (Fig. 1). The reactions

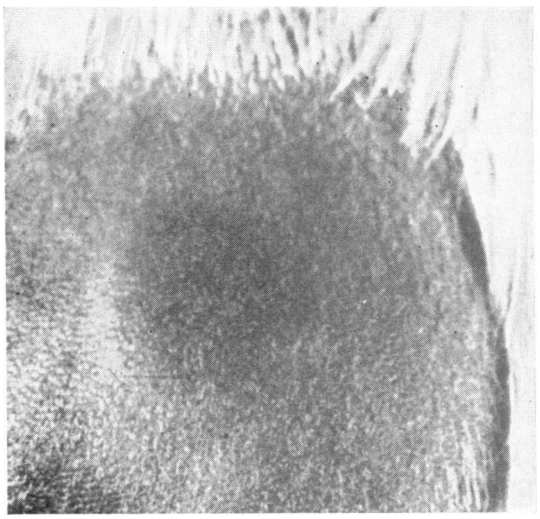

FIG. 1. Cutaneous reaction in recipient 24 hours after intravenous injection of testis-sensitized lymph-node cells and intradermal challenge with 0.5 mg. purified testis antigen.

were maximum at 24 hours and visible for 48–72 hours, some necrosis of the skin occurring after this time in the stronger reactions.

In the third experiment, varying amounts of sensitized cells (400, 800 and 1600 million) from the same pool were transferred into recipients, each of which was injected with two doses of antigen (0.05 and 0.5 mg.). Sixteen hundred million cells gave a graded response with the two doses of antigen, 800 million gave a response with the larger, but not with the smaller, dose and 400 million failed to give a response with either dose.

These results are summarized in Table 1. It is seen that a positive reaction could be obtained by the intraperitoneal transfer method provided that about 800 million cells were given. The responses were graded in accordance with the number of cells and the dose of antigen used.

TABLE 1

DELAYED RESPONSE TO TESTIS ANTIGEN IN RECIPIENTS OF TESTIS-SENSITIZED LYMPH-NODE CELLS INJECTED INTRAPERITONEALLY

(Antigen injected intracutaneously 48 hours after cell transfer)

Experiment number	*Number of cells*	*Mg. antigen*	*Response (mm. × mm.) 24 hours after antigen*
1	520×10^6	1	2 × 2
		0.25	2 × 2
		0.05	2 × 2
		Saline	2 × 2
	520×10^6	1	6 × 5
		0.25	4 × 3
		0.05	3 × 3
		Saline	3 × 2
2	1940×10^6	0.5	12 × 16 } Right flank
		0.05	10 × 10 } Right flank
		Saline	2 × 2 } Right flank
		0.5	10 × 13 } Left flank
		0.05	7 × 7 } Left flank
		Saline	— } Left flank
3	400×10^6	0.5	2 × 1
		0.05	—
	800×10^6	0.5	6 × 7
		0.05	—
	1600×10^6	0.5	10 × 11
		0.05	5 × 5

INTRAVENOUS TRANSFER OF CELLS

In another series cells from testis-sensitized guinea pigs were transferred intravenously into recipients. Females were used both as donors and as recipients. The antigen (0.5 mg. purified testis extract) was injected intracutaneously immediately after the transfer of the cells and the readings were taken after 24 hours. The results of this series are summarized in Table 2.

In the first experiment, two recipients received 350 and 490 million cells respectively. They were challenged with single doses of antigen and both gave good responses.

In the second experiment an attempt at a dose-response curve was made by giving 150, 300 and 520 million cells each to one recipient. Only the recipient of 520 million cells responded to the antigen, and a second and third challenge gave diminishing responses. The other recipients failed to respond even to the first injection.

In the third experiment the plan of the second was repeated with a larger number of cells (225, 450 and 900 million). Each recipient received two challenging doses of antigen, one on each flank. The responses were graded in accordance with the number of cells, as shown in Table 2. The magnitude of the response, relative to the number of cells, was somewhat less than in the earlier experiments, suggesting that the double challenge had diminished individual responses.

Fig. 2 shows a comparison of the effectiveness of intraperitoneal and intravenous transfers. The intravenous method is more sensitive, especially if the recipient is challenged

TABLE 2

DELAYED RESPONSE TO 0.5 MG. TESTIS ANTIGEN IN RECIPIENTS OF TESTIS-SENSITIZED LYMPH-NODE CELLS INJECTED INTRAVENOUSLY

Experiment number	*Number of cells*	*Time of challenge after cell injection*	*Response (mm. × mm.) 24 hours after antigen*
1	350×10^6	15 minutes	7 × 9
	490×10^6	15 minutes	7 × 11
2	150×10^6	15 minutes	—
	300×10^6	15 minutes	2 × 2
	520×10^6	15 minutes 20 hours 44 hours	9 × 14 6 × 10 3 × 3
3	225×10^6	15 minutes 15 minutes	2 × 2 Left flank — Right flank
	450×10^6	15 minutes 15 minutes	5 × 5 Left flank 2 × 2 Right flank
	900×10^6	15 minutes 15 minutes	10 × 13 Left flank 9 × 13 Right flank

with a single dose of antigen; under these conditions 400 to 500 million cells give a definite reaction. With two simultaneous challenging doses, the critical number lies somewhere between 450 and 900 million cells. Intraperitoneal injection gave smaller responses for a given number of cells and produced a flatter dose-response curve.

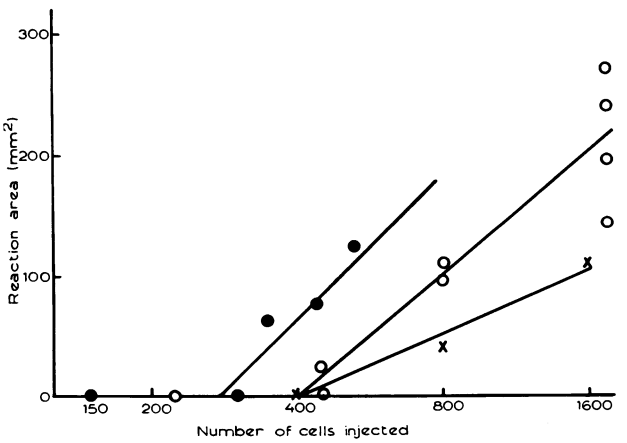

FIG. 2. Relationship between the number of testis-sensitized cells transferred and the skin reactions in normal recipients 24 hours after intracutaneous injection of 0.5 mg. testis antigen. Antigen was injected immediately after the cells in the intravenous transfers and 48 hours after the cells in the intraperitoneal transfers. (1) Intravenous transfers with a single challenge (●), (2) intravenous transfers with two simultaneous challenges (○), (3) intraperitoneal transfers with a single challenge (×).

EFFECT OF DURATION OF DONOR SENSITIZATION ON THE SKIN REACTIONS OF DONORS AND RECIPIENTS OF SENSITIZED CELLS

Skin Reactions of Actively Sensitized Animals

Eight female guinea pigs were sensitized and challenged, two at a time, 3, 6, 12 and 24 days after sensitization. Skin reactions were read 2, 4, 6, 8, 24 and 48 hours after injection of the challenge dose. Fig. 3 shows the time course of these reactions, each point

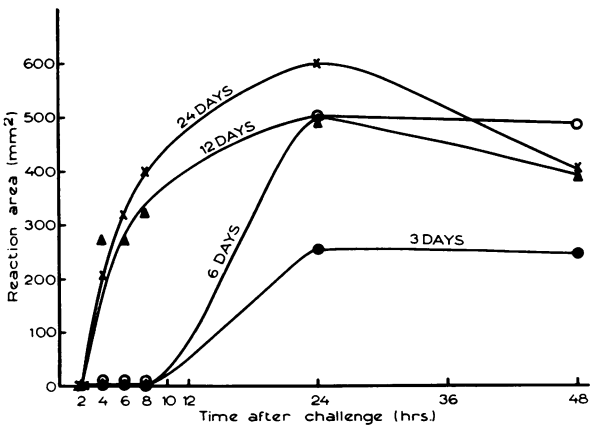

FIG. 3. Time course of skin reactions to 0.5 mg. testis antigen in guinea pigs after different periods of active sensitization. Each curve represents the mean reaction of two guinea pigs challenged for the first time and sensitized for: 3 (●), 6 (○), 12 (×) and 24 (▲) days.

representing the mean reaction area of the two guinea pigs tested. Three and 6 days after sensitization, the reactions were of a pure delayed character, hardly visible up to 8 hours, maximum after about 24 hours and persisting for 48 hours; 12 and 24 days after sensitization the reactions began earlier at 4 hours, continued to increase up to 24 hours, and persisted for 48 hours but were then reduced in size. These latter reactions seemed to consist of two components. They were at first ill-defined, pink and somewhat oedematous, whereas 24 hours after challenge they became flatter, red and more clearly defined, similar to the delayed reactions seen earlier.

A second set of eight guinea pigs gave similar results.

Skin Reactions in Cell Recipients

(a) *Intravenous transfers.* Batches of female guinea pigs were actively sensitized and used 3, 6, 12 and 24 days later for cell transfers into single female recipients. The latter came from the same batch as the donors and each received a standard dose of 1400 million cells. In the last two experiments an excess of cells was available and a further recipient was, therefore, injected in each case with 700 million cells. The challenging dose of antigen (0.5 mg. purified testis extract) was given intracutaneously immediately after cell transfer, and was repeated daily until the responses became negligible. Normal controls were injected with the antigen at the same times as the cell recipients.

The results of this series are shown in Table 3. Lymph-node cells obtained 3 days after sensitization were not capable of transferring the delayed reactivity. The recipient

TABLE 3

DELAYED RESPONSE TO TESTIS ANTIGEN IN RECIPIENTS OF TESTIS-SENSITIZED LYMPH-NODE CELLS INJECTED INTRAVENOUSLY, IN RELATION TO THE DURATION OF SENSITIZATION OF CELL DONORS

Duration of sensitization (days)	*Time of challenge after cell injection*	*Response (mm. × mm.) 24 hours after antigen*		
		1400 × 10^6 cells	*700 × 10^6 cells*	*Control*
3	15 minutes	—		2 × 2
	24 hours	—		—
6	15 minutes	15 × 17		Trace
	24 hours	14 × 20		2 × 1
	48 hours	14 × 20		—
	72 hours	15 × 20		—
	6 days	2 × 3		2 × 2
12	15 minutes	12 × 10	10 × 8	—
	24 hours	8 × 9	—	—
	48 hours	5 × 4		—
24	15 minutes	11 × 14 9 × 11	7 × 7	3 × 3
	24 hours	9 × 11 —	—	—
	48 hours	5 × 6 —		

of cells obtained 6 days after sensitization gave a large response to an immediate challenge; similar responses were obtained in this animal by further injection of antigen 1, 2 and 3 days after cell transfer, although a challenge on the sixth day after transfer was negative. Recipients of cells obtained after 12 and 24 days sensitization gave conspicuous responses to an immediate challenge. The reactions in these animals were not as large as that seen in the recipient of cells sensitized for 6 days and further challenges gave diminishing responses. The reaction in the recipient of cells after 6 days sensitization was not only exceptionally large but it was also qualitatively different from the rest. There was evidence of an early Arthus-like reaction component and the reaction was greatly reduced in size 48 hours after challenge. Reactions in the other recipients were of a typical delayed type.

(b) *Intracutaneous transfers.* Lymph-node cells from a group of donors were used for intracutaneous transfer into female recipients 3, 6, 12 and 24 days after sensitization. Two donors were used for each sensitization period and two recipients for each donor. Each recipient was given four injections, two containing 5 million cells and two containing 10 million cells, with and without 0.5 mg. antigen. Each injection was made in a total volume of 0.1 ml. Tyrode solution. The 24-hour reactions were scored on an arbitrary scale by a panel of observers. The results of these experiments are shown in Fig. 4 and Table 4. As previously reported (Baum *et al.*, 1961) intracutaneous injection of testis-sensitized cells alone produced a reaction nearly as large as that of the cells injected together with antigen. However, in the present experiments it was found that addition of antigen produced a significant increase in the reaction due to 5 million cells ($P<0.01$) but not in that due to 10 million cells ($P>0.05$).

Fig. 4 shows the difference in cutaneous response of 5 million cells injected with and without antigen in relation to time of sensitization of cell donors. In spite of a great deal of scatter, there is a tendency for this difference to be more positive with increasing time of sensitization.

TABLE 4

CUTANEOUS REACTIONS PRODUCED IN NORMAL ANIMALS BY INTRACUTANEOUS INJECTION OF 5 AND 10 MILLION TESTIS-SENSITIZED LYMPH-NODE CELLS WITH AND WITHOUT ANTIGEN. CELLS WERE OBTAINED FROM GUINEA PIGS SENSITIZED FOR 3, 6, 12 AND 24 DAYS AND REACTIONS WERE SCORED ON AN ARBITRARY SCALE BY A PANEL OF OBSERVERS AFTER 24 HOURS

Time of sensitization (days)	5×10^6 *cells*			10×10^6 *cells*		
	Alone	*With antigen*	*Difference due to antigen*	*Alone*	*With antigen*	*Difference due to antigen*
3	0.5	0.5	0	1.3	1.3	0
	0.3	1.4	+1.1	0.8	1.6	+0.8
	1.3	1.1	−0.2	1.7	1.5	−0.2
	1.2	0.9	−0.3	1.6	1.3	−0.3
6	0.5	0.9	+0.4	1.6	1.3	−0.3
	0.1	0.1	0	0	0	0
	0.9	1.6	+0.7	1.2	0.7	−0.5
	1.2	1.5	+0.3	1.5	2.4	+0.9
12	0.6	1.0	+0.4	1.7	1.9	+0.2
	0.4	0.5	+0.1	0.5	0.5	0
	0.7	1.3	+0.6	1.1	1.3	+0.2
	1.3	1.3	0	1.8	1.8	0
24	0.5	1.0	+0.5	0.5	1.0	+0.5
	0.5	1.0	+0.5	1.2	1.8	+0.6
	1.2	1.0	−0.2	1.8	2.3	+0.5
	0.5	0.8	+0.3	1.2	1.0	−0.2
	0.5	2.0	+1.5	1.5	2.0	+0.5
	1.2	1.8	+0.6	1.8	2.2	+0.4

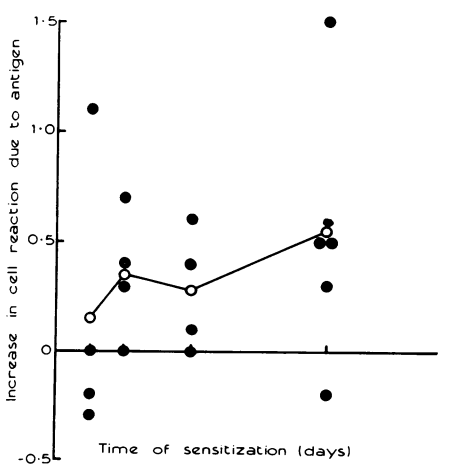

FIG. 4. Effect of antigen on the reaction due to 5 million cells injected intracutaneously into a normal recipient in relation to time of sensitization of the cell donors. Mean values for each sensitization period are indicated by (○). The ordinate represents the difference between reactions due to cells with antigen and cells alone.

DISCUSSION

These experiments show that lymph-node cells from guinea pigs sensitized with an extract of homologous testis and Freund's adjuvant will transmit delayed skin hypersensitivity to normal recipients. The experiments were carried out on females in order to avoid interference by a pre-existing testis antigen in both donors and recipients. Cells transferred intraperitoneally as well as those transferred intravenously induced delayed skin sensitization. The latter route was the more effective, as about half the number of cells were required to produce the same degree of sensitivity in the recipient. With the intravenous procedure there was no delay between cell transfer and intracutaneous challenge; possibly in the more protracted intraperitoneal procedure some of the cells were destroyed by an early homograft reaction (Boyse, 1959). Intradermal transfers of cells also gave positive results which tended to be masked by unspecific reactions produced by the intracutaneously injected cells.

The sensitization of lymph-node cells developed rapidly: strong reactions were obtained in recipients by injecting cells from donors which had been sensitized for only 6 days. On the whole the skin reactions of the donors and recipients corresponded but the donor reactions were generally larger and they started sooner. Fig. 5 shows that 3 days after

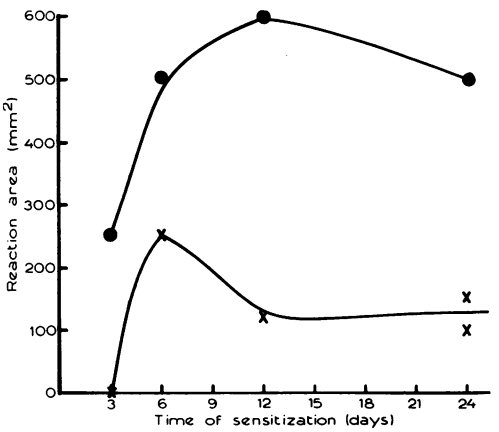

FIG. 5. Comparison of delayed response to 0.5 mg. testis antigen in actively sensitized animals (•) and in recipients of 1400 million sensitized lymph-node cells injected intravenously (×) in relation to time of sensitization of donors.

active sensitization when the transfer reaction was negative, the actively sensitized donors already gave appreciable responses; later on the donor reactions were greater than those in recipients in spite of the large number of cells transferred into each recipient from as many as twelve donors.

As a rule, the skin reactions in both actively and passively sensitized animals were of the delayed type; they had a latent period of 6 to 8 hours, were maximum after 24 hours and persisted more or less unchanged for 48 hours. In some instances, reactions of the immediate type were also apparent. The actively sensitized animals at first gave pure delayed reactions, but after about 12 days they began to show mixed reactions which appeared as early as 4 hours after injection of the challenging dose of antigen. By contrast, the only instance of a response of the immediate type in a recipient was seen in an animal which had been injected with cells from donors sensitized for 6 days, who themselves gave

a pure delayed response. Bauer and Stone (1961) have observed that in the course of tuberculin sensitization, the cell population of the lymph nodes draining the injection site changes; in the early stages the cells have the character of plasma cells. It is possible that a transfer of cells such as plasma cells, involved in the production of humoral antibody (Fagraeus, 1958), may result in a release in the recipient of antibody capable of producing a reaction of the immediate type. This hypothesis could be tested by finding out whether isolated skin or lung from recipients of sensitized cells become passively sensitized and release histamine when treated with testis antigen (Baum *et al.*, 1961).

A passive transfer of hypersensitivity by means of cells has previously been described for various delayed reactions, such as the tuberculin reaction, but not so far for a sensitization by an autoantigen or organ-specific antigen as defined by Voisin (1958). Our experiments show no evidence of any fundamental difference between the skin responses after transferring testis and tuberculin cells. Some of the points of similarity between these two reactions are discussed below.

(1) Metaxas and Metaxas-Buehler (1955) came to the conclusion that no latent period was required for the establishment of skin reactivity after passive sensitization with tuberculin cells by the intravenous route. This is equally true of testis cells; the latent periods of the skin reactions of actively and passively sensitized animals after challenge with antigen were similar. The skin reactions of recipients took about 24 hours to develop irrespective of the route of cell injection, in spite of the fact that in the intraperitoneal method an interval of 48 hours elapsed between cell transfer and administration of the antigen, whereas in the intravenous method cells and antigen were injected almost simultaneously.

(2) In agreement with the findings of Metaxas *et al.* (1955) on tuberculin cells, the reactivity of the skin after passive sensitization with testis cells declined at a rapid rate. The speed of establishment and decline of the hypersensitivity suggests that the antigen in these cases reacts directly with the transferred sensitized cells which presumably do not proliferate in the recipient. Our animals were not inbred; the injection of sensitized cells into inbred recipients may result in a more complicated situation in which the transferred cells proliferate (Bauer *et al.*, 1961) and perhaps also produce humoral antibody.

(3) Testis cells as well as tuberculin cells produce reactions when they are injected intradermally together with the appropriate antigen, but both also produce unspecific skin irritation. We have found (in unpublished experiments) that tuberculin-sensitized cells give much stronger reactions with the appropriate antigen than testis cells and are therefore more suitable for intradermal transfer experiments.

(4) Multiple injections of antigen into a recipient guinea pig produce smaller responses than single injections after the transfer of either tuberculin or testis cells. This supports the hypothesis that the antigen reacts with a limited number of sensitized cells rather than with an antibody already fixed to the skin.

No comparative studies are available of the rate of sensitization of lymph-node cells to tuberculin or testis antigen. It would be interesting to know whether the sensitization to tuberculin develops at the same rapid rate as to testis antigen and, more generally, whether the two kinds of sensitization can coexist within the same cell.

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