CYTOTOXICITY MEDIATED BY SOLUBLE ANTIGEN AND LYMPHOCYTES IN DELAYED HYPERSENSITIVITY

II. Correlation of the In Vitro Response with Skin Reactivity.*

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In earlier publications (1, 2) we described the death of rat fibroblasts in the presence of specific antigen and lymph node cells from rats sensitized to either tuberculoprotein, egg albumin, or bovine gamma globulin. The cytotoxic effect is antigen specific, has a delayed time course, and does not appear to require complement. A close similarity has been established, in terms of antigen concentration, relative cell number, and time course, between this system and other in vitro systems currently accepted as manifestations of delayed hypersensitivity. These include inhibition of macrophage migration (3–8), blast transformation (9, 10), autoallergic cytotoxicity (11, 12), and homograft cytotoxicity (13, 14). In the present paper we present evidence which suggests a relationship between our system and in vivo manifestations of cellular hypersensitivity.

Materials and Methods

The methods were essentially those described in the previous paper (2).

Antigens.—Antigens included tuberculoprotein (PPD), obtained from Parke, Davis & Co., Detroit, Michigan; five times crystalline chicken egg albumin (EA), from Nutritional Biochemicals Corp., Cleveland, Ohio; and human serum albumin (HSA), from Upjohn Co., Kalamazoo, Mich. Hapten-protein conjugates, prepared and kindly provided by Dr. Fred Kantor of the Department of Medicine, Yale University, included picryl ovalbumin (Pic EA) (27.5 groups/mole), picryl human serum albumin (Pic HSA) (39.1 groups/mole), and picryl guinea pig albumin (Pic GPA) (38.5 groups/mole). The EA and HSA used in these preparations were obtained from Worthington Biochemical Corp., Freehold, N.J., and the Red Cross respectively.

Sensitization.—The techniques of sensitization were those described previously. Doses of

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300 μg of tubercle bacilli (Tbc) and conjugates were used. EA was used for primary sensitization at a dose of 100 μg. A saline solution of 500 μg EA was employed for booster injection.

Skin Tests.—Skin test doses of 50 μg PPD in diluent or 30 μg of other protein antigens and conjugates in saline were injected in the shaved flank. Reactions were observed 3 hr (Arthus) and 24 hr (delayed) later. The diameter was measured, and the degree of induration graded on an arbitrary scale of 0 to ++++.

Cytotoxicity Assays.—For cytotoxicity assay, 150–200 × 10^5 lymph node cells were added to rat fibroblasts in the presence of antigen (2). Lymph node cells were obtained 9 days after sensitization in all experiments except those indicated in Table IV. In the latter, the cytotoxic effect of cells from animals exhibiting strong delayed sensitization was compared with that of cells from rats sensitized in such a way as to exhibit weak delayed skin reactivity and correspondingly stronger immediate hypersensitivity. All cultures were trypsinized and harvested

TABLE I

Specificity of Skin Reactions in Lewis Rats

Sensitizing antigen	No. of animals	Skin test antigen	Average diameter of skin reaction	
			3 hr	24 hr
			mm	*mm*
Pic EA	3	Pic EA	3 ±0 SD	14 ±0.8 SD
		EA	5.3 ±0.9	10 ±1.5
		Pic GPA	3.6 ±0.25	0
Pic HSA	4	Pic HSA	8.25 ±1.5	25 ±3.5
		HSA	0	16.3 ±2.2
		Pic GPA	8.5 ±0.9	9 ±0.7

Animals sensitized with 300 μg antigen in complete adjuvant in footpad and skin tested at 9 days with 30 μg of each test material.

SD, standard deviation of the mean.

72 hr after the addition of lymph node cells and antigen and the surviving fibroblasts were enumerated in an electronic particle counter (2).

RESULTS

Groups of adult male Lewis rats were sensitized with Pic EA or Pic HSA and skin tested at 9 days with several different materials. No Arthus (3 hr) reactions could be elicited in the former group even with homologous antigen (Table I). Those sensitized with Pic HSA showed mild, hapten-specific reactions. The delayed (24 hr) reactions, in each instance, were strongest against the conjugate of hapten with homologous carrier, less with the homologous carrier alone, and absent or very weak with hapten conjugated to heterologous carrier. Lymph node cells, from animals similarly sensitized but not skin tested, produced a definite cytotoxic effect on syngeneic fibroblasts with homologous conjugate, a significant but reduced effect with homologous carrier alone, and no effect with the specific hapten conjugated to a heterologous protein (Table II, Fig. 1).

Lewis and BN rats sensitized with tubercle bacilli in oil were skin tested with PPD at 9, 21, and 35 days. There was no significant difference in the 3 hr reactions between the two strains (Fig. 2). However, Lewis rats showed more intense *delayed* skin reactivity than BN rats, reflected in significantly greater diameter (Fig. 3) and induration of the 24 hr skin reactions. The Lewis reactions were all graded ++++, and many were necrotic; BN reactions were either +++ or ++. This difference was matched by a distinct difference in the degree of fibroblast damage produced in the presence of antigen by sensitized Lewis as contrasted with BN lymph node cells. Fibroblast survival was significantly

TABLE II

Carrier Specificity of Cytotoxic Effect Mediated by Lymph Node Cells from Rats Sensitized with Conjugates

Exp.	Sensitizing antigen	Substance added to culture, 15 μg/ml	Surviving fibroblasts in presence of		Survival (b/a × 100)
			a	b	
			Normal lymph node cells	Sensitized lymph node cells	
			× 10^5	× 10^5	
1	Pic EA	Pic EA	9.3	3.2	34
		EA	9.9	7.5	75
		Pic HSA	9.9	8.7	88
		No addition	—	—	—
2	Pic HSA	Pic HSA	6.4	0.93	14
		HSA	6.9	4.2	61
		Pic GPA	5.1	5.3	104
		No addition	6.6	6.2	94

lower in the presence of the former, whether syngeneic or allogeneic target cells were used (Table III).

In rats sensitized with tubercle bacilli in oil, delayed reactivity to tubecrulin is intense at 9 days and waning at 24 days (Table IV). Lymph node cells harvested at 9 days, from rats which had been sensitized but not skin tested, produced a more intense cytotoxic effect than those taken from littermates at 24 days. Similarly, in animals sensitized with EA, there was intense delayed reactivity by 9 days, while Arthus reactions, presumably reflecting the level of circulating antibody, remained mild. Following an intravenous booster injection of EA in saline late in immunization, there was a diminution in delayed sensitivity accompanied by intensification of Arthus reactivity. Again cells from littermates of rats with intense delayed skin reactions gave a strong cytotoxic effect; those from animals with poor delayed sensitivity gave a weaker effect (Table IV).

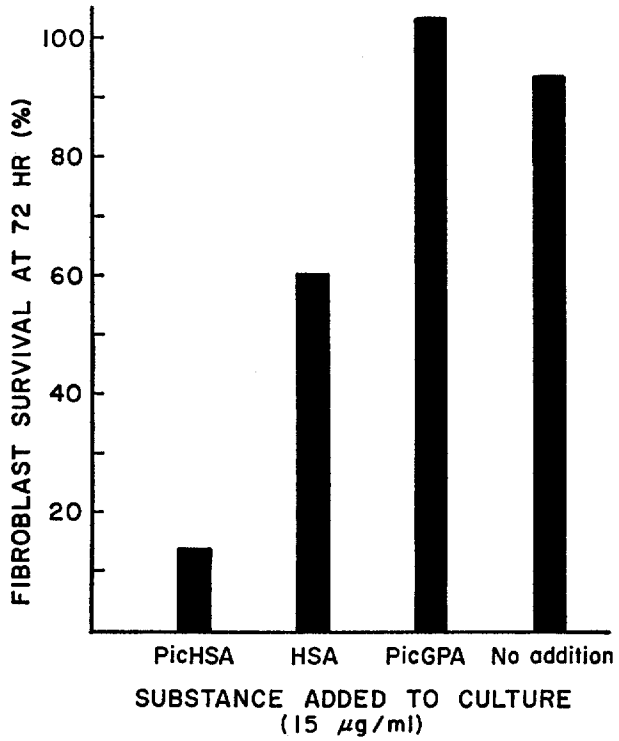

FIG. 1. Carrier specificity of the cytotoxic effect of sensitized lymph node cells. 150×10^5 cells from Lewis rats sensitized with picryl HSA were added to each culture of Lewis fibroblasts, together with 15 μg/ml of test substance.

DISCUSSION

In the preceding paper (2) many points of similarity were established between fibroblast killing, mediated by soluble antigen and sensitized lymphocytes, and other in vitro phenomena commonly considered manifestations of delayed hypersensitivity. Suggestive evidence that this cytotoxicity phenomenon is a true correlate of delayed hypersensitivity is provided by its carrier specificity (15). In vitro observations paralleled skin tests, positive reactions being elicited by Pic EA or EA but not by Pic HSA in sensitivity to Pic EA, and by Pic HSA or HSA and not by Pic GPA in sensitivity to Pic HSA.

In comparable studies with other systems, Carpenter and Brandriss (16) found that migration of cells from spleen and lung explants of guinea pigs sensitized with picrylated guinea pig skin extract was inhibited by the immunizing antigen, minimally by Pic HSA, picrylgelatin, or skin extract alone, and not by Pic EA. David et al. (5) found that DNP-GPA inhibited the migration of peritoneal exudate cells from DNP-GPA immunized guinea pigs; DNP-BGG did not inhibit nor did the carrier alone. Since

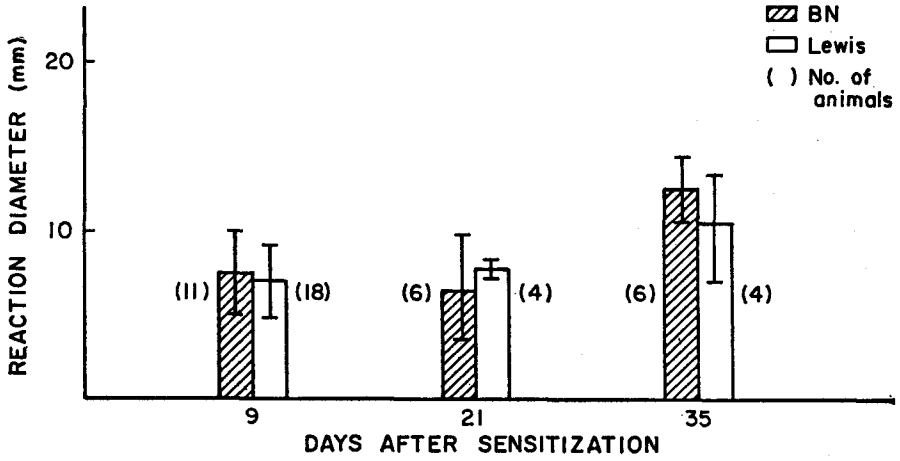

FIG. 2. Comparison of reactions in tuberculin-sensitized BN and Lewis rats 3 hr after skin test with 50 μg PPD. The reactions of the two strains were not significantly different 9 days after sensitization (P = 0.70–0.80), 21 days after sensitization (P = 0.50–0.60), or 35 days after sensitization (P = 0.20–0.30).

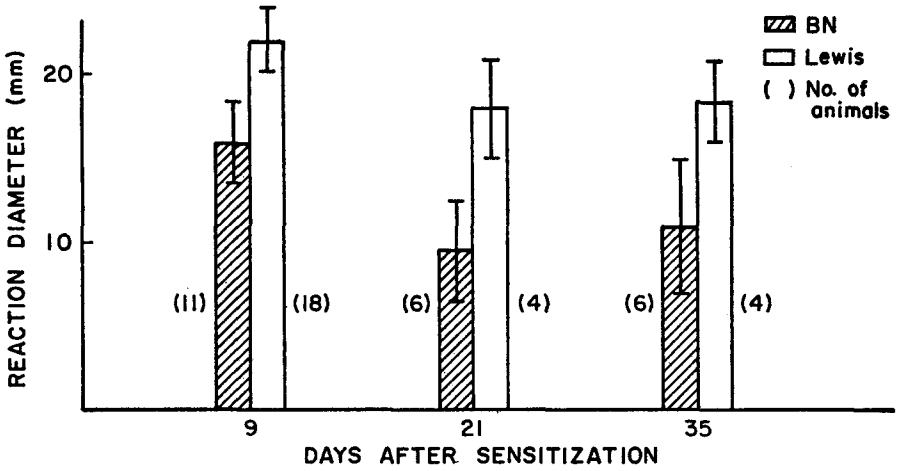

FIG. 3. Comparison of reactions in tuberculin-sensitized BN and Lewis rats 24 hours after skin test with 50 μg PPD. The reactions of Lewis rats were significantly larger than those of BN rats 9 days after sensitization (P = < 0.001), 21 days after sensitization (P = 0.001–0.01), and 35 days after sensitization (P = 0.02–0.05).

GPA is a guinea pig protein, however, this may not have been a sufficient test of the carrier's ability to elicit a reaction (17). Cells from animals sensitized to DNP-BGG were inhibited by DNP-BGG but not DNP-GPA, DNP-BSA, or DNP-EA; the homologous carrier was not tested alone. Mills (9) obtained strong blast transformation

of leukocytes from guinea pigs sensitized to DNP-BGG or DNP-EA with the immunizing conjugate, none with the hapten conjugated to heterologous carrier, and an insignificant response with homologous carrier alone. Oppenheim et al. (10), with lymphocytes from guinea pigs immunized with guinea pig albumin orthanilic acid, obtained blast transformation with the homologous antigen but not with the unconjugated hapten, guinea pig albumin alone, or the hapten coupled to a heterologous protein.

Our results remain qualified since the possibility that free carrier protein may have been present in the original immunizing solutions was not completely eliminated.

TABLE III

Comparison of the Cytotoxic Effect of Sensitized Lymph Node Cells from BN and Lewis Rats in the Presence of Antigen

Exp.	Source of fibroblasts	Lewis lymph node cells			BN lymph node cells		
		Surviving fibroblasts		Survival (b/a × 100)	Surviving fibroblasts		Survival (d/c × 100)
		Normal lymph node cells	Sensitized lymph node cells		Normal lymph node cells	Sensitized lymph node cells	
		a	b		c	d	
		× 10^5	× 10^5	%	× 10^5	× 10^5	%
3	Lewis	6.2	1.6	26	6.7	4.6	69
4	Lewis	6.4	2.1	32	7.2	5.3	74
5	Lewis	6.6	2.6	40	6.4	5.0	79
6	BN	11.6	1.8	16	10.98	5.95	55
7	BN	7.6	2.7	35	7.4	4.9	66
8	BN	7.4	1.9	26	6.2	4.3	69
Average				29.1±7.7	$P<0.001$		68.6±7.4

12.5 or 25 μg PPD/ml added to each culture.
P, calculated by T test.

The parallel between delayed skin reactivity and the cytotoxic effect of sensitized lymph node cells in the presence of antigen was shown to extend to the difference between Lewis and BN rats. Our observations suggest one mechanism which may underlie genetically determined differences in the ability to develop delayed sensitivity (18). In the present case, the difference depends on differences in the specifically sensitized lymphocytes themselves rather than in target cells or other nonspecific elements of the reaction such as the vascular supply. The cytotoxic effect is apparently not correlated with antibody formation since 3 hr reactions in the two rat strains were almost identical. In experiments designed to investigate the effect of a booster injection of antigen on

various parameters of the immune response, the cytotoxic capacity of lymph node cells again paralleled delayed reactivity, diminishing after the booster at a time when Arthus reactivity was increasing. These results are similar to those obtained by David et al. in control studies of the macrophage migration inhibition system (5). Wilson (14) and David et al. (3–5) have ruled out the possibility that cytophilic antibody participates either in inhibiting macrophage migration or in homograft cytotoxicity. Comparable controls have not yet been

TABLE IV

Correlation between Skin Reactivity and Cytotoxic Effectiveness of Lymph Node Cells under Different Conditions of Sensitization

Exp.	Sensitizing antigen	Time after initial sensitization	Skin Reactions*				Surviving fibroblasts		Survival (b/a × 100)
			3 hr		24 hr		Normal lymph node cells	Sensitized lymph node cells	
			Diameter	Degree	Diameter	Degree	a	b	
		days	*mm*		*mm*		× 10^5	× 10^5	%
9	Tbc	9	12	++	23	++++	5.75	1.26	22
	Tbc	24	9	+	16	+	5.75	4.11	71
10	EA	9	14	+	21	++++	11.66	4.39	37
		200‡	14	++	3	0	11.66	8.02	69
11	EA	9	13	+	30	++++	9.47	3.53	37
	EA	40§	21	+++	18	++	9.47	5.41	57

Cultures harvested 72 hr after addition of 150 × 10^5 and 12.5 μg PPD/ml (Exp. 1) or 25 μg EA/ml (Exp. 2 and 3).

* Average diameters in groups of 1–3 rats. Degree of induration estimated subjectively on a scale of 0–++++.

‡ Intravenous injection of 500 μg EA in saline 2 days before assay.

§ Intravenous injection of 500 μg EA in saline 4 days before assay.

carried out with the system described here. However, the fact that heat-inactivated serum was used throughout these experiments militates against the possibility that cytotoxicity might have been mediated by antibody and complement.

The description of a new phenomenon related to cellular hypersensitivity justifies a renewed attempt to account for the properties of delayed reactions in vivo in terms of what has been observed in vitro. Delayed skin reactions share characteristic morphologic features with experimental autoallergic lesions and tissue transplants undergoing rejection (20, 19). Each consists of a rapidly progressing perivenous mononuclear cell infiltration. The cell infiltrate may contain a few specifically sensitized cells (21), but the majority are nonspecific

phagocytic mononuclears (22) which have been shown to come from the bone marrow by way of the blood stream (23–25). They are comparable to histiocytes or macrophages which appear at sites of nonspecific inflammation (25, 26) and to the peritoneal exudate mononuclears used in many in vitro experiments (27, 3–8). There is parenchymal damage in the zone of cell infiltration (19) which may affect epidermis in the lesion of contact allergy (28), such tissue elements as myelin and thyroid acinar epithelium in the autoallergies (29–31), and all the cellular elements of grafted tissues (32, 33). Vascular necrosis in intense lesions may also represent a form of parenchymal damage produced by the infiltrating cells (34).

The in vitro observations suggest that in vivo specific lymphocytes (whether remaining in the vessel lumen or extravascular) react with antigen at the site and release a mediator which can damage cells (6–8, 35). This mediator may affect vascular endothelium and conceivably parenchymal elements near the vessel. Circulating monocytes acted upon by the mediator become activated (27) and sticky (36, 3) (stickiness is itself a manifestation of activation [37]. These changes in the monocytes and possibly the vessel wall result in sticking of the monocytes to the endothelium and diapedesis. Alternatively the damage of endothelium and parenchyma may result in tissue breakdown products which induce diapedesis comparable to that following nonspecific (e.g. thermal) injury (38, 39). Finally, hydrolases released by the activated cells may be responsible for parenchymal damage, since such cells are themselves highly cytopathogenic[1] (40). The relative roles in tissue destruction of the lymphocytic mediator and of macrophage enzymes remains to be defined.

SUMMARY

Damage of rat embryo fibroblasts in the presence of sensitized lymph node cells reacting with specific antigen was shown to be closely correlated with delayed hypersensitivity in the animals from which the lymph node cells were taken. The phenomenon was not correlated with Arthus reactivity. In animals sensitized with picryl conjugates of ovalbumin or human serum albumin, skin reactivity and the in vitro cytotoxic effect could be elicited only with the homologous conjugate or the protein carrier alone and not with picryl conjugates of heterologous proteins. Lewis rats developed more intense delayed sensitivity than BN rats, and Lewis lymph node cells were correspondingly more effective in producing specific damage of both syngeneic and allogeneic fibroblasts.

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