ANALYSIS OF THE EXPERIMENTAL LESION OF CONNECTIVE TISSUE PRODUCED BY A COMPLEX OF C POLYSACCHARIDE FROM GROUP A STREPTOCOCCI

II. Influence of Age and Hypersensitivity*

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A chronic, remittent, multinodular lesion of the connective tissue of rabbits is induced with a peptide–C polysaccharide complex from the cell walls of beta hemolytic streptococci (1). Some form of hypersensitivity is the most obvious explanation to invoke for the mechanism of this lesion, but histological, immunological, and enzymatic analyses do not support this concept (2–4).

If the toxic manifestations of a bacterial component are dependent upon a mechanism of hypersensitivity, susceptibility of animals should increase with age. This is based upon the assumption that the immunological competence of the neonatal animal is not well developed, and that continued exposure to cross-reactive bacterial antigens in the environment will increase the hypersensitive state of the animal as it ages. Toxicity of endotoxins from Gram-negative bacteria, for example, increases directly with age, and most of the toxic effect has an allergic basis (5).

The influence of age of the animal on the chronic relapsing lesion produced by streptococcal components was studied to obtain further evidence of the role of hypersensitivity in this reaction. The age distribution of susceptibility indicates that the chronic nodular lesion involves some physiological maturation besides the immune response. The experiments were designed to follow the evolution of the allergic and of the relapsing nodular reactions, from neonatal through adult life, in rabbits sensitized by specific active or passive sensitization, or sensitized by natural contact with microbial antigens in their environment, cross-reactive with Group A streptococcal components.

Materials and Methods

Streptococcal Cell Extract.—A sterile extract of a Type 3, Group A streptococcus was prepared as described elsewhere (1). A washed cell suspension was disrupted by sonic vibration,

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centrifuged at 27,000 *G*, the supernatant dialyzed against water, filtered through a Millipore HA filter, and lyophilized. This crude extract contained the mucopeptide–C polysaccharide cell wall fragments which produce the relapsing nodular lesion, as well as other soluble constituents of the cell. This preparation had at least 9 antigenic components shown by immunodiffusion and was used instead of more highly purified cell wall fragments to enable maximum detection of allergic reactions to the streptococcal cell. A single intradermal injection of 1.0 mg in 0.1 ml sterile saline was given in the upper flank of rabbits varying in age from 1 day to 6 months.

Animals.—Pregnant New Zealand white rabbits were obtained from a local breeder about 3 weeks before parturition and kept in individual cages with the usual standards of a clean animal house. The litters were weaned to individual cages at 5 to 6 weeks.

Immune Serum and Spleen Cells.—Adult rabbits were injected intradermally at 4 sites on two occasions 1 month apart with a heat-killed suspension of washed Group A, Type 3 streptococci. One month later they were bled from the heart. As expected with this method of immunization, the serum contained no antibodies against C polysaccharide or cell walls which could be detected by a ring precipitin test with these antigens. The spleens from 3 animals were removed and minced in Hanks' solution. The suspension was shaken on a vortex mixer for 15 minutes and decanted through sterile gauze. The residue was suspended and filtered a second time and the cell suspensions pooled. These contained an average of 4.2×10^7 cells per ml.

Allergic Reactions.—Reactions assumed to reflect a spectrum of hypersensitivity were recorded as the area in square millimeters of erythema and edema at 3 hours, 24 hours, and 3 days after injection. The early reactions, reaching a peak at about 3 or 24 hours, consisted of a red, soft edema characteristic of a moderate Arthus reaction. The early response subsided within 48 hours and was followed in the older animals by a firm, indurated, erythematous area which reached its peak between 2 and 4 days after injection. None of the reactions proceeded to necrosis.

Since a crude cell extract was employed, it is not known whether the shift in phases of maximum response with age reflects a change in the type of immune response to one predominant antigen, or qualitatively different responses to different antigens.

Multinodular Relapsing Lesions.—This reaction was clearly distinguishable in time and gross appearance from the earlier hypersensitivity responses. The nodular lesion appeared 4 to 16 days after injection, and the process of remission and relapse continued for as long as 80 days after the single intradermal injection. A detailed description of the gross and microscopic features of this lesion has been given (3). The index is a measure of this reaction and is obtained by dividing the maximum area of the lesion in square millimeters by the time in days required for gross appearance of the nodules (1). A comparison of nodular lesions in 2 litters of the same age in which the litter mates were all injected at the same time gives an indication of the variance observed within and between litters. The mean index of each litter was 156 and 96 with a standard error of 58 and 57 respectively. There is no difference in the variance of the litters, and the difference between the means is not significant at the 0.10 level of confidence.

RESULTS

Effect of Age of the Animal.—In the first experiment (Table I, Fig. 1) the 37 rabbits under 8 weeks of age came from 8 litters. Litter mates were injected at ages varying from 1 day to 4 weeks; older rabbits were obtained directly from the breeder.

No allergic response was apparent in the gross in rabbits under 4 weeks of age. By about 4 weeks transient erythema and edema appeared within 4 hours after injection and largely subsided by 24 hours. As the rabbits increased in age, or if specifically sensitized by a prior intradermal injection, the early reaction

became more persistent, and the response was more prominent 24 hours after injection. The "delayed" reaction at about 72 hours was apparent only in the older animals.

It was of great interest that the nodular lesions developed in neonatal animals which showed no type of allergic reaction in the gross. Furthermore, in older animals there was no correlation between area of early allergic reaction and the index of the nodular lesions (Fig. 2). Since there is a limited surface area on a rabbit under 4 weeks of age the moderate nodular lesion index in these groups is probably misleading. The difference between groups I, II, III, and IV is not

TABLE I

Hypersensitivity Reactions and Relapsing Nodular Lesions in Rabbits Injected at Various Ages

Group	Age at time of injection	Mean allergic lesion*			Nodular lesions				
		4 hrs.	24 hrs.	72 hrs.	Mean index‡	No. neg.	Relapse		
							1	2	3
I	1 to 3 days	0	0	0	18	5/12	5/10	2	1
II	5 to 9 days	0	0	0	18	0/5	5/5	4	0
III	10 to 16 days	0	0	0	58	0/5	3/5	0	0
IV	17 to 30 days	5	0	0	62	1/5	2/4	0	0
V	4 wks.	479	43	0	126	0/10	8/8	2	0
VI	8 wks.	579	263	0	145	0/4	2/4	0	0
VII	Approximately 12 wks.	419	257	19	118	0/4	3/4	0	0
VIII	Over 6 months	602	948	151	36	5/10	0/10	0	0
IX	Over 7 months (second injection of group VIII)	265	633	403	27	2/5	1/5	0	0

* Average area of erythema and edema in mm^2 at 4, 24, and 72 hours after injection.

‡ Index is the maximum area of the lesion in mm^2 divided by the time in days for gross appearance.

significant at the 0.05 level of confidence. Groups V and VI are significantly different from group I at the 0.001 level and from group VIII at the 0.05 level. It is apparent from Fig. 1 that the chronic multinodular lesion is most severe during the period of most active growth. This is in contrast to older animals which display the most extensive early and delayed allergic reactions, but very moderate nodular lesions and rare relapses. The severity of the nodular lesion begins to subside somewhat before the growth rate decreases and at about the same time the delayed reaction appears.

It is noteworthy that the incidence of relapses, which is the most unique feature of this lesion, is greater in younger than in older animals. The recurrence of relapses within one animal is also seen only in very young rabbits with this dose.

Repeated Injections.—The question of the relationship of nodular lesions to

hypersensitivity was pursued by injecting the same rabbits at 3 age levels. Eleven animals who were given the first injection of streptococcal extract in the neonatal period (1 to 30 days old) were injected again at 8 weeks and given a third injection at 6 months.

Table II shows that neither the latent period between injection and appearance of nodules, nor the severity of the nodular lesion, is related to the number

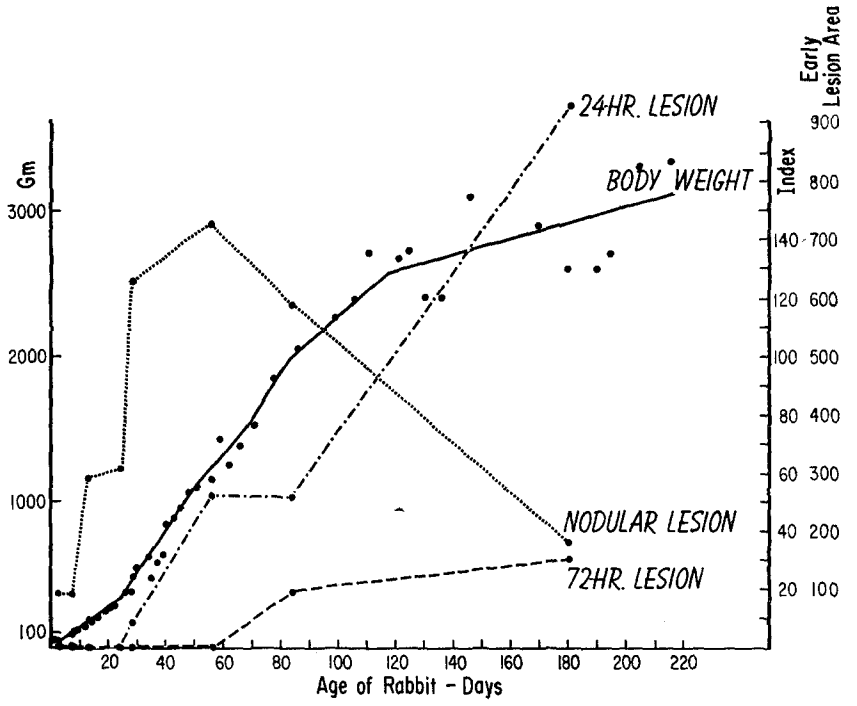

FIG. 1. Variation of 24-hour allergic lesion (mm^2), 72-hour allergic lesion (mm^2), nodular lesion index, and body weight (gm) with age of rabbit at time of injection.

of injections or the intensity of the early allergic reactions. Although the nodular reaction increases at the second injection it is again less intense at the third injection while the allergic reaction has increased. Since these results are comparable to those in Table I where the animals representing each age group were given single injections, the decreased nodular reaction at the third injection probably reflects a response characteristic of animals of this age rather than a specific immune resistance. Also, in a previous experiment (3) adult rabbits given repeated injections over a shorter age span did not show significantly different nodular reactions.

Passive Transfer of Hypersensitivity.—While it is apparent that the development of the nodular lesion is not dependent upon a recognized mechanism of

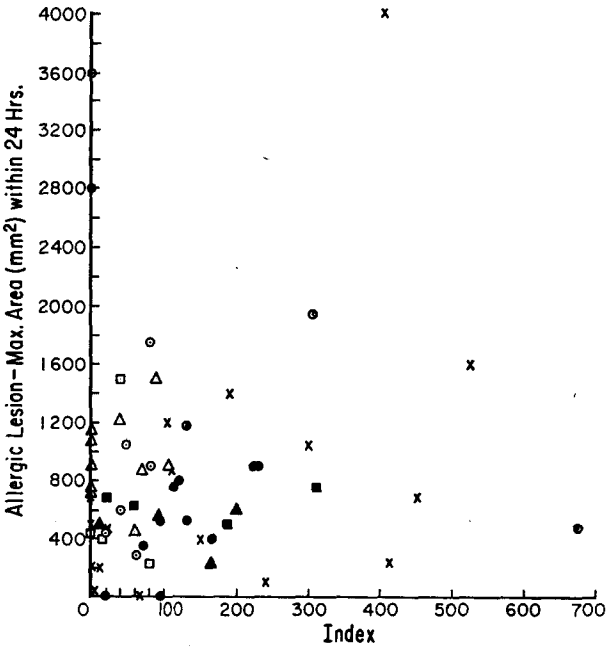

FIG. 2. Correlation of early allergic skin reaction (maximum area of erythema and edema within 24 hours after injection) with nodular lesion index. 4 weeks old, first injection, ●; 8 weeks old, first injection, ■; 8 weeks old, second injection, ×; 3 months old, first injection, ▲; 6 months old, first injection, △; 7 months old, second injection, □; 6 months old, third injection, ⊙.

TABLE II

Effect of Repeated Injection of Streptococcal Extract at Three Age Levels

Tissue response	First injection (1 to 30 days old)	Second injection (8 wks. old)	Third injection (6 months old)
Nodular lesion:			
Latent period, *days*	6.6	4.8	10.6
Area, (mm^2)	307	905	725
Index	62	219	77
Relapse	7/11	4/11	1/11
Allergic lesion: 24 hrs., (mm^2)	0	1264	1554

hypersensitivity, there is still some question whether the decreasing severity of the nodular lesion in older rabbits is related to the increasing allergic response. To clarify this point an attempt was made to transfer hypersensitivity from old rabbits to neonatal animals.

The neonatal animal will support antibody production by passively trans-

ferred immune cells, although there is disagreement regarding the relative amount of antibody produced (6, 7). Sterzl (8) was unsuccessful in attempts to transfer delayed hypersensitivity to neonates, but the evidence presented here indicates a very moderate passive transfer can be achieved.

Serum and a suspension of spleen cells in Hanks' solution were obtained from 3 adult rabbits which had been specifically sensitized by intradermal injection of heat-killed Group A streptococcal cells. Litter mates from 3 litters were divided into three groups. One group received an intraperitoneal injection of approximately 8.2 x 10^7 spleen cells in 2.0 ml. of Hanks' solution. A second group was injected intraperitoneally with 2.0 ml of serum, and a third group was given 2.0 ml of Hanks' solution intraperitoneally. The ages of 3 litters were 3, 5, and 7 days. Forty-eight hours later each animal was injected intradermally with 1.0 mg of streptococcal extract. None of the animals showed a visible reaction at

TABLE III

Effect of Passive Transfer of Spleen Cells and Serum from Sensitized Adult Rabbits to Neonatal Rabbits

Intraperitoneal injection	Allergic lesions, mean area				Nodular lesions			
	4 hrs.	24 hrs.	48 hrs.	72 hrs.	Latency	Area	Index	Relapse
	mm^2	mm^2	mm^2	mm^2	*days*	mm^2		
Spleen cell suspension	0	36	16	12	5.7	229	54	5/7
Serum	0	15	4	6	5.5	314	69	2/4
Hanks' solution	0	5	0	4	6.5	297	56	1/4

4 hours. The mean areas of erythema and edema recorded at 24, 48, and 72 hours are given in Table III. These values are negligible for the groups given serum or Hanks' solution. The values for the group given spleen cells are small but significantly greater than the other two groups, indicating that a degree of hypersensitivity has been successfully transferred to this group of neonatal animals. There is no difference between the nodular lesion production in these three groups, nor can the incidence of relapses be considered different. We conclude, therefore, that the factors responsible for the hypersensitivity reactions are not contributing to the decreased severity of nodular lesions in older animals.

DISCUSSION

These observations demonstrate that a well characterized component of Group A streptococci can produce a chronic, relapsing, nodular lesion of connective tissue independent of the hypersensitive state of the animal. The lesion is produced in neonatal rabbits whose immunological competence is not well developed. As shown here and elsewhere (3), the latent period before appear-

ance of nodules is not shortened, the severity of the lesion and incidence of relapses is not increased, and the basic histological features are not changed, following second and third injections of the streptococcal material.

An age distribution of susceptibility was also demonstrated: the lesion being most severe in rapidly growing animals, and less severe in older rabbits at an age when the presumably allergic early reaction was greatest. This observation is significant in view of current studies which indicate that the development of the evolving lesion can be related to quantitative changes of connective tissue elements (9).

SUMMARY

Rabbits presumably sensitized to components of the streptococcal cell by natural contact with cross-reactive microbial antigens in their environment, display distinctive phases of evolving allergic response to an extract of streptococcal cells. Animals under 4 weeks of age show no gross allergic skin reaction; this is followed successively by age periods in which allergic reactions become most prominent at 4 hours, 23 hours, or 2 to 4 days after injection.

A relapsing nodular lesion, produced by cell wall components in the cell extract, occurs in neonatal rabbits which display no hypersensitivity, reaches a peak response in young animals during the period of most active growth, and decreases in severity in older rabbits while the degree of hypersensitivity is increasing. Relapses occur with greater frequency in neonatal than in older rabbits. Passive transfer of spleen cells or serum from sensitized adult rabbits has no influence on the nodular reaction in neonatal animals.

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