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Immunological Response to Glomerular Basement Membrane and Streptococcal Antigens in Immunized Rabbits

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Summary. Cellular and humoral responses were investigated following human glomerular basement membrane and streptococcal Type 12 membrane injections in rabbits. Using the leucocyte migration inhibition test and double diffusion in agar gel, cross-reactivity between the antigens was evident, though neither antigen induced significant proteinuria or nephritis in the dosages given.

INTRODUCTION

Studies of experimental glomerulonephritis (Unanue and Dixon, 1967) have established that glomerular injury can result from two distinct mechanisms, the glomerular localization of antigen–antibody complexes, or the deposition of anti-glomerular basement membrane antibodies. The role of cell-mediated immunity however is still not understood, although several laboratories have reported evidence of this type of immune mechanism in patients with nephritis (Bendixen, 1968; Rocklin, Lewis and David, 1970; Zabriskie, Lewshenia, Möller, Wehle and Falk, 1970; Dardenne, Zabriskie and Bach, 1972; Mallick, Williams, McFarlane, Orr, Taylor and Williams, 1972).

The purpose of this investigation was to assess evidence for an immunological response to both glomerular basement membrane (GBM) and streptococcal antigens in rabbits. The leucocyte migration inhibition test of Bendixen and Søberg (1969) was used as an *in vitro* measure of cellular response, and circulating (humoral) antibodies were assayed by immunodiffusion in agar gel (Ouchterlony, 1958).

MATERIALS AND METHODS

Glomerular basement membrane (GBM) preparation

Human cadaver kidneys were frozen and stored at −20° in airtight plastic bags until used. Glomeruli were isolated from the cortex by the method of Spiro (Beisswenger and Spiro, 1973) and glomerular basement membranes were prepared by ultrasonication in 1·0 M saline (Beisswenger and Spiro, 1973).

Soluble GBM antigens were obtained by enzymic digestion of the ultrasonicated sus-

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pension (25 mg/ml) with collagenase (Worthington) (1 per cent) at 37° for 24 hours in borate buffer (pH 7·0).

Streptococcal membrane antigens

Two streptococcal membrane antigens were kindly supplied by Dr J. Zabriskie (Rockefeller University, New York): a group A Type 12 and an S43/192. Soluble antigen was extracted with sodium deoxycholate at 37°. The concentration of protein used was 5 mg/ml with 15 mg/ml of deoxycholate in the solution. The cholate was removed from the extract by filtration through Sephadex G-25.

Active immunization of rabbits

All rabbits used were New Zealand white males averaging 4 kg in weight. Twenty animals were tested, eight being immunized with GBM and six with streptococcal T12 membrane antigen. The remaining six animals acted as controls.

The immunization procedure involved five weekly intramuscular injections in Freund's complete adjuvant, following a control period of 2–5 weeks. Two different doses of insoluble GBM were given, 2·5 mg (two animals) and 10 mg (six animals); and two doses of insoluble streptoccal T12 membrane antigen: 3 mg (two animals) and 5 mg (four animals). Of the six control animals, four were not given any injections, whilst two had saline in Freund's complete adjuvant weekly for 5 weeks.

Leucocyte migration inhibition test (LMI)

This test was performed according to the method of Bendixen and Søberg (1969) with minor modifications.

Fifteen to 20 ml of venous blood was collected weekly from the ear of each rabbit. Six millilitres was allowed to clot and the serum stored for subsequent antibody determination. The remainder was collected into 10 per cent EDTA (0·5 ml EDTA per 10 ml of blood). The red blood cells were sedimented by mixing the blood with an equal volume of 2 per cent gelatin in 0·9 per cent saline in a measuring cylinder and incubating at 37° for 20 minutes. The cylinder was stood at an angle of 45° for 15 minutes and then upright for a further 5 minutes. The leucocyte-rich plasma was removed and centrifuged at 2000 rev/min for 5 minutes. The cell pellet was freed from contaminating red cells by lysis with 0·83 per cent ammonium chloride for 4 minutes and then centrifuged as above. The leucocyte pellet was then washed with 0·9 per cent saline and resuspended in TC 199 medium supplemented with 10 per cent heat-inactivated normal rabbit serum (HINRS) so that the final concentration of cells was 20×10^6/ml.

Capillary tubes (Hawksley, microhaematocrit) were filled with the cell suspension, sealed at one end (Hawksley, Cristaseal) and centrifuged at 1000 rev/min for 5 minutes. The tubes were cut 1 mm below the cell–fluid interface and the cell pellet positioned by means of silicone grease (BDH) in a leucocyte migration chamber (Sterilin Ltd). For each rabbit, one series of three chambers was filled with TC 199 + 10 per cent HINRS alone, and the other series with medium plus antigens. The chambers were sealed with glass coverslips (Gallenkamp, Chance 22 mm) and incubated horizontally at 37° for 24 hours. The fan-like pattern of migration was projected onto a screen and the areas drawn. These areas were then measured by planimetry and the effect of an antigen on cell migration was expressed as a percentage of the migration without antigen as follows: migration index = [(mean of three migrations in antigen)/(mean of three migrations in TC 199)] × 100.

In order to establish the range of migration without antigen, the three individual migrations in TC 199 were expressed as a percentage of the mean migration in TC 199 for each animal for each week. The mean and standard deviation of these results were calculated at the end of the experiment and the levels of $\pm 2 \times$ s.d. were taken as the 'normal range' of migration for the leucocytes of that animal.

Antigens

The antigens employed in the LMI test are listed in Table 1. All were diluted in TC 199+10 per cent HINRS. Where the optimum dose of antigen was unknown, several dilutions were tested. The doses of the insoluble antigens are expressed as wet weight per millilitre and the soluble antigens as micrograms per millilitre of protein (Folin Ciocalteau Method). BSA and PPD are expressed as dry weight per millilitre.

Immunodiffusion

Reactions were carried out in 1 per cent agar made up in phosphate-buffered saline (pH 7·4).

Double diffusion was performed on 8 cm^2 glass slides using well cutters of 8 mm in diameter.

RESULTS

The cellular and humoral response was apparently unaffected by the variations in injection concentration. Similarly the response of the control rabbits injected with saline did not differ from those having no injections.

Figs 1 and 2 show the inhibitions observed in two animals which showed good immuno-

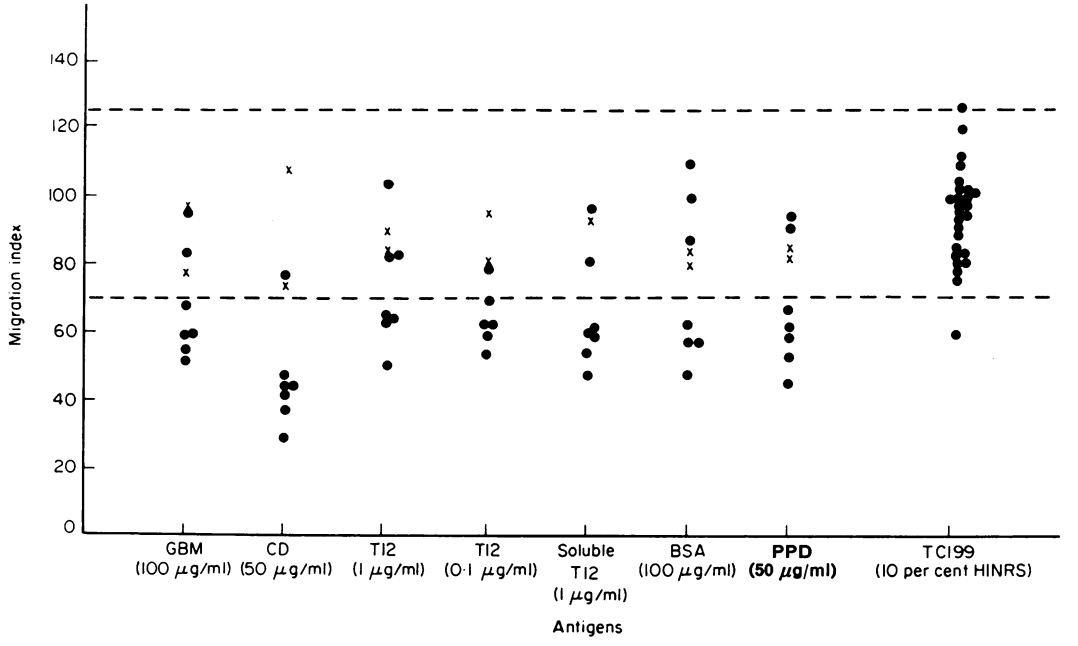

FIG. 1. The 'effect' of antigens on the migration of leucocytes from a GBM-injected rabbit. (●) Post-injection. (×) Pre-injection.

logical responses following GBM and streptococcal T12 injections. The control observations for each animal represent all the migrations in medium only, both from pre-injected and sensitized cells, and the levels of $\pm 2 \times$ s.d. are marked.

In both figures, inhibition is most marked with collagenase digest (50 μg/ml) (CD) but is clearly present for a series of other antigens at various concentrations. The positive inhibition to PPD after injection in Freund's complete adjuvant, which is more marked in the GBM-immunized rabbit, supports the view that migration inhibition does indicate leucocyte sensitization.

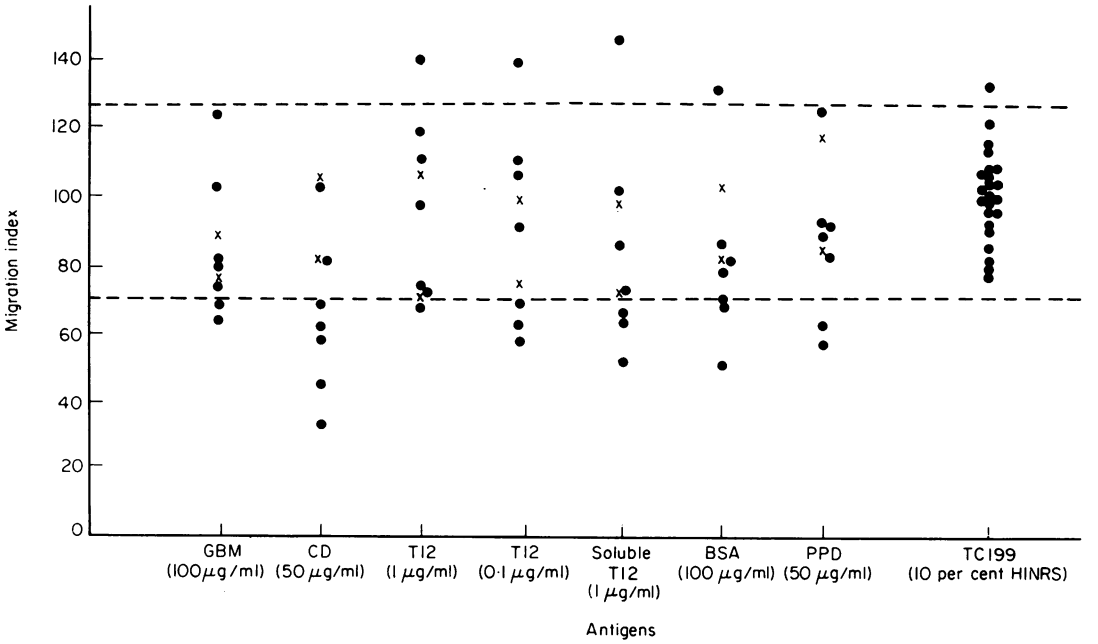

FIG. 2. The 'effect' of antigens on the migration of leucocytes from a streptococcal T12-injected rabbit.

Unfortunately some animals responded less satisfactorily than those represented in Figs 1 and 2 and so the overall results for the eight GBM and six streptococcal T12 immunized animals are difficult to express statistically. Table 1 compares the responses of the control and sensitized rabbits to the various antigens employed. The italicized results indicate an increased inhibition after injection.

In comparison to the control animals, the rabbits injected with GBM show a marked increase in inhibition with GBM (100 μg/ml), T12 (1·0 μg/ml and 0·1 μg/ml), soluble T12, BSA and PPD. A slight increase of inhibition occurred with CD (50 μg/ml) and S43 (0·01 μg/ml). The rabbits injected with streptococcal T12 antigen responded to the same antigens (with the exception of S43 (0·01 μg/ml)) but to different degrees. The GBM-injected animals showed a greater degree of inhibition with GBM (100 μg/ml), T12 (1·0 μg/ml) and PPD, than did the streptococcal injected groups.

It would seem that the optimum dose of insoluble GBM for the *in vitro* system is 100 μg/ml, as the results for 50 and 1 μg/ml indicate a decreased inhibition in the immunized animals when compared with the controls. The same phenomenon occurs with CD

TABLE 1

DATA ON CONTROL AND IMMUNIZED RABBITS

Antigen concentration (μg/ml)	Control rabbits* Observations Inhibited	Total	Percentage inhibition	GBM-injected rabbits† Observations Inhibited	Total	Percentage inhibition	T12-injected rabbits‡ Observations Inhibited	Total	Percentage inhibition
GBM									
100	2	55	3·6	10	43	*23·3*	4	31	*12·9*
50	4	12	33·3	4	16	25·0	1	15	6·7
1	5	12	41·7	1	16	6·3	0	15	0
CD									
50	17	67	25·4	17	60	*28·3*	22	47	*46·8*
1	5	13	38·5	0	16	0	0	16	0
T12									
1	1	24	4·2	6	14	*42·9*	3	13	*23·1*
0·1	2	56	3·6	8	44	*18·2*	8	31	*25·8*
0·01	5	32	15·6	1	29	3·5	2	18	11·1
Soluble T12									
1	1	24	4·2	6	14	*42·9*	4	13	*30·8*
BSA									
100	3	24	12·5	6	14	*42·9*	5	13	*38·5*
PPD									
50	1	24	4·2	8	14	*57·1*	3	13	*23·1*
S43									
0·1	3	13	23·1	2	16	12·5	1	17	5·9
0·01	2	9	22·2	3	12	*25·0*	1	11	9·1

* Results derived from six control rabbits and pre-injection phases of test rabbits.
† Results derived from eight rabbits.
‡ Results derived from six rabbits.

(1 μg/ml), T12 (0·01 μg/ml) and S43 (0·1 μg/ml). However, S43 (0·01 μg/ml) shows an increased inhibition in the GBM rabbits, but a decrease in the T12 animals, when compared with the control result.

The increased inhibition with BSA in both the GBM and T12 immunized animals would seem to indicate some non-specific sensitization. This assumption is strengthened by the fact that the percentage inhibition is of the same order in both test groups.

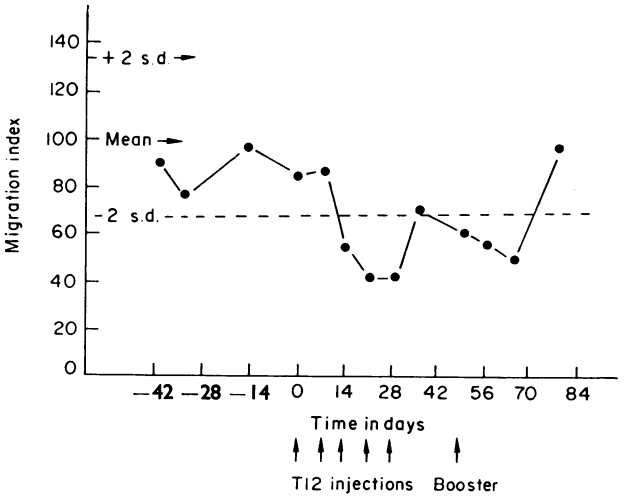

FIG. 3. Migration indices of a streptococcal T12-injected rabbit challenged with CD (50 μg/ml).

The timing of the cellular response varies slightly between antigens, but in general is strongest between 14 and 50 days after the first injection. After this period, the migration indices return to normal, but a secondary response lasting approximately 2 weeks can be initiated by giving a booster injection (Fig. 3).

HUMORAL ANTIBODIES

Both the GBM and streptococcal T12 injected animals gave rise to high titres (1:64 and 1:128) of circulating antibody against the injected antigen on double diffusion agar plates. More important, however, was a complete cross-reaction in all injected animals between GBM and T12 antigens. Those animals injected with GBM showed lower titres against T12 than did the T12-injected animals against GBM. No control animal showed any evidence of reaction against GBM or T12 and it was noted that the reaction disappeared fairly rapidly after the last injection.

DISCUSSION

The experiments showed some similarity of response both in cellular sensitization and in the production of circulating antibody between GBM antigen and streptococcal T12 membrane antigen in rabbits. The response is, however, of relatively short duration and within the limits of dosage used, independent of the amount of antigen. No animal developed proteinuria, or any evidence of nephritis at subsequent autopsy and it is clear that in the rabbit, neither human GBM antigen nor streptococcal T12 membrane antigen will produce permanent glomerular damage. The remarkable similarity of response to the two antigenic stimuli, however, does suggest some common structure between GBM and certain streptococcal membranes and further study is now being directed to the characterization of the common antigen and also to finding a more satisfactory animal model for the study of this reaction.

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