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Immunological unresponsiveness during induction of experimental autoimmune orchitis in guinea-pigs: studies *in vivo* and *in vitro*

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Summary. Groups of male and female guinea-pigs were immunized with homologous sperm derived from testis (TS) or epididyimis (ES) in Freund's complete adjuvant (FCA). *In vivo* investigations included skin tests at 2 weeks and development of aspermatogenesis (testis weight) at 4 weeks; *in vitro* assays were inhibition of migration of peritoneal exudate cells (PEC) and culture of blood leucocytes (lymphocyte transformation) at weekly intervals after immunization. Antigens used were heat-treated extracts of sperm used for immunization (BTS, BES); cells were also cultured simultaneously with PPD. Skin tests revealed anergy in males as compared with females: a larger quantity of antigen which caused partial unresponsiveness in females, caused profound unresponsiveness in males although the aspermatogenesis was less severe. *In vitro* tests also showed anergy during the active stages of the orchitis. This was non-specific for PEC (specific unresponsiveness was not excluded), but blood leucocytes showed only specific unresponsiveness (to BES). These and previous studies suggest that the unresponsiveness results from a desensitisation by sperm antigens released during the development of aspermatogenesis.

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INTRODUCTION

There is evidence that skin responsiveness is reduced in both clinical and experimental autoimmune disorders. Varying degrees of anergy have been observed in patients with systemic lupus erythematosus (Block, Gibbs, Stevens & Shulman, 1968; Horwitz & Cousar, 1975) and rheumatoid arthritis (Lockshin, 1976). In experimental autoimmunity the skin response may be reduced (Wasserman & Packalin, 1965; Salvin & Liauw, 1968; Eylar, Caccam, Jackson, Westall & Robinson, 1969; McFarlin, Hsu, Slemenda, Chou & Kibler, 1975), or delayed in development (Brown, Glynn & Holborow, 1963; Marcus, Soffer, Ben-David, Peleg & Nebel, 1973) and *in vitro* responses also, are late to develop (Clinton & Weigle, 1972; Marcus *et al.*, 1973; Weigle & Romball, 1975).

We had observed a reduced skin response in male, as compared with female guinea-pigs immunized with epididymal sperm (ES) in FCA (Muir, Turk & Hanley, 1976). We wondered if this difference might be caused by the autoimmune orchitis, for the females showed no evidence of an autoimmune disorder. Therefore, to elucidate mechanisms by which the efferent responses might be suppressed

in autoimmunity, we investigated in detail the efferent responses of the guinea-pigs both *in vivo* by skin tests and development of aspermatogenesis, and *in vitro* by inhibition of migration of PEC and transformation of peripheral blood leucocytes. The results establish that an immunological unresponsiveness occurs during the development of the aspermatogenesis and that the most probable mechanism is a desensitization caused by release of sperm antigens.

MATERIALS AND METHODS

Animals

Outbred Hartley strain guinea-pigs, weighing 525–725 g at the time of immunization were used for all experiments. They were obtained from four sources – Royal College of Surgeons animal colony; A. Tuck & Sons, Rayleigh, Essex; Bantin & Kingman Ltd., Aldbrough, Hull; Allington Farm, Porton Down, Wilts.

Antigens

Freeze-dried epididymal sperm (ES) and the heat-treated extract (BES) were prepared as described previously (Muir *et al.*, 1976).

Testis sperm (TS) was prepared by crushing frozen guinea-pig testes and filtering the tissue through nylon gauze with PBS. The resulting single cell suspension of the contents of seminiferous tubules was centrifuged and the sediment (TS), freeze-dried. For the heat-treated extract, a large quantity of TS was boiled in PBS for 30 min; homogenized by hand; centrifuged at 105,000 *g* for 3 h and the sediment discarded. The supernatant was concentrated by freeze-drying to a protein concentration of approximately 8 mg/ml (Biuret method), dialysed against PBS, sterilised by filtration (Millipore 0·2μ) and stored at –20°.

PPD (Central Veterinary Laboratory, Weybridge) was dialysed against PBS and diluted with PBS as necessary.

Ovalbumin, OA (Miles-Seravac, Maidenhead) was dissolved in PBS and the solution sterilized by filtration.

Immunization

For each animal 10 mg ES or 5, 10 or 20 mg TS was suspended in 0·25 ml distilled water and emulsified with 0·25 ml FCA (H37 Ra). For some animals 5μg OA was used instead of ES. The final volume, 0·5 ml, was injected into the hind footpads and nuchal region. Control animals were immunized with FCA emulsified with PBS without antigen.

Skin tests

These were done as described previously (Muir *et al.*, 1976) using BES or BTS, 100 μg in 0·1 ml. The response was read at 5, 24 and 48 h after intradermal injection.

Testis weight and histology

This was done as described previously (Muir *et al.*, 1976).

Inhibition of migration of PEC

This was similar to the method of David, Al-Askari, Lawrence & Thomas (1964), using PEC induced by injection of paraffin 5 days previously. Cells were cultured in supplemented MEM (Wellcome), with 10% foetal calf serum. Antigen concentrations used were BES 100 μg and PPD 50 μg per ml medium. Migrations were projected and traced out after 20 h incubation at 37°. At least four capillaries were cultured with each concentration of antigen. At least one control animal, immunized with FCA only, was set up in parallel with each group. Results were expressed as the percentage inhibition of migration calculated as follows:

$$\%\,\mathrm{MI} = \left(\frac{\text{Mean area of migration with antigen}}{\text{Mean area of migration without antigen}}\right) \times 100$$

Lymphocyte transformation

This was done as described previously (Muir, Turk & Hanley, 1977) using blood taken by cardiac puncture. Cells were cultured with 5, 50 and 200 μg BES and 50 μg PPD per ml of medium for 96 h.

RESULTS

Skin reactions

Figure 1 shows the characteristics of the skin response to BES and PPD, 2 weeks after immunization with 10 mg ES in FCA. We had established previously that in males, the skin response was minimal at this time (Muir *et al.*, 1976). In females, the skin response to BES did not differ from that to PPD in animals immunized similarly; the males showed little skin response.

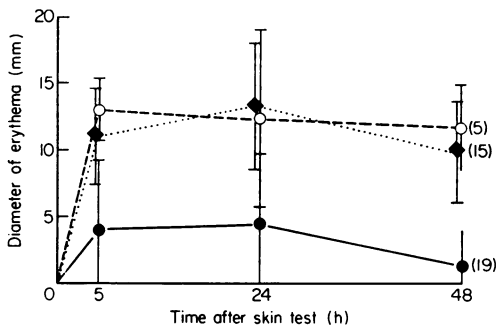

Figure 1. Skin response to BES and PPD. Guinea-pigs immunized with 10 mg ES in FCA and skin tested at 2 weeks. Numbers in parentheses indicate number of animals in group. Separate groups of females for each antigen. Males, (solid line); females (BES) (dotted line); females PPD), (dashed line).

As the ES was in short supply, we investigated the use of TS as immunizing antigen, this being more freely available. The characteristics of the skin response to BTS in guinea-pigs immunized with 10 mg TS in FCA, is shown in Fig. 2. In both groups of animals a mixed Arthus and delayed hypersensitivity reaction is shown. The delayed hypersensitivity component is again significantly reduced in the males.

We wished to investigate further the nature of the unresponsiveness in the males. Animals were immunized with 2, 10 or 20 mg TS in FCA and skin tested with BTS at 2 weeks. Table 1 shows that 20 mg TS induced significant unresponsiveness to BTS in both females and males, but in the latter

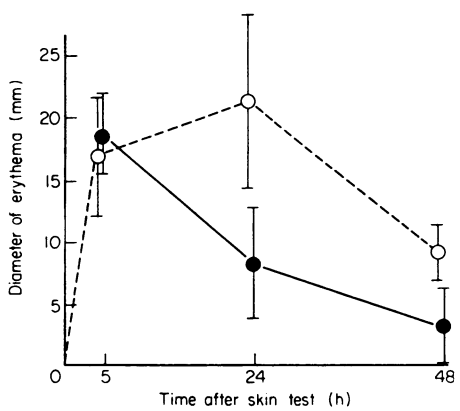

Figure 2. Skin response to BTS. Groups of five guinea-pigs immunized with TS in FCA and skin tested at 2 weeks. Males, (solid line); females, (dashed line).

Table 1 Effect of quantity of TS used for immunization. 24 h ski response to BTS at 2 weeks

Quantity of TS used (mg)	Diameter of erythema (mm)	
	Male	Female
2 (5)	5·8 ± 5·7	—
10 (5)	12·6 ± 1·1	21·4 ± 6·9
20 (9)	N M R	10·8 ± 8·1

All values are ± s.d. of mean. NMR = No measurable response. In parentheses are numbers of animals in each group. A highly significant difference ($P < 0.01$) is shown between groups of females and between groups immunized with 10 and 20 mg TS. Difference between groups of males immunized with 2 and 10 mg TS is not significant.

it was severe. As 2 mg TS caused little or no aspermatogenesis (see below), it was concluded that this was too small a quantity of antigen.

Aspermatogenesis

Table 2 shows the aspermatogenesis induced after immunization with the various quantities of TS. The value of testis weight as a criterion of aspermatogenesis had been established previously (Muir *et al.*, 1976). It is seen that animals immunized with 10 mg TS developed significantly more aspermatogenesis than those immunized with 2 or 20 mg. The aspermatogenesis was also confirmed histologically (appearances not shown).

Inhibition of migration of PEC

Table 3 shows the effect of culturing PEC from guinea-pigs immunized with ES in FCA with PPD at intervals after immunization. When the PEC were from females, significant inhibition of migration (> 25%) was shown in all cultures, by 2 weeks after immunization. However, when the PEC were from males, inhibition of migration was not significant until 4 weeks. The difference in migration between PEC from males at 2 weeks and from females at 2–3 weeks is highly significant ($P < 0.01$). No consistent *in vitro* response to BES was shown by PEC from either males or females.

Lymphocyte transformation

Table 4 shows the blood leucocyte cultures from female guinea-pigs immunized with ES in FCA, at 3 to 5 weeks after immunization. The cells from all animals showed significant uptake of [^{3}H]-thymidine

Table 2. Aspermatogenesis after immunization with TS in FCA. Testis weight at 4 weeks.

Quantity of TS used for immunization (mg)	No. of animals in group	Mean testis weight ± SD (g)	*P* value (compared with normal testes)
2	5	2·19 ± 0·86	>0·05
10	10	1·79 ± 0·78	<0·001
20	10	2·33 ± 0·88	<0·01

For estimating *P*, each group was compared with testis weight from fifteen normal guinea-pigs of comparable weight (mean = 3·22 ± 0·62g) by Students' *t* test. Difference between groups immunized with 10 and 20 mg TS was highly significant by the paired *t* test ($P < 0{\cdot}001$).

Table 3. Inhibition of migration of PEC: cultures with PPD. Guinea-pigs immunized with ES in FCA.

Weeks after immunization	Sex	No. animals in group	% MI ± S.D.
1	F	5	5 ± 5
	M	5	—12 ± 23
2	F	4	53 ± 21
	M	9	13 ± 33
3	F	5	45 ± 15
4	M	5	54 ± 15
6	M	4	52 ± 8

A value of MI less than 0, indicates enhanced migration. The difference of MI between males at 2 weeks and females at 2–3 weeks is highly significant ($P < 0{\cdot}01$).

with both BES and PPD. The result of leucocyte cultures from male guinea-pigs is shown in Figs 3 and 4. Significant uptake of [^{3}H]-thymidine was shown by all cultures with PPD (S.R. >2·0), at 3 or more weeks after immunization, although the mean response was maximal by 2 weeks. However, the cultures with BES (Fig. 4) show that the mean S.R. of each group increased up to 4 weeks after immunization and did not exceed the level of non-specific stimulation (S.R. 2·5) until this time. Thus, the cultures with BES show that the development of *in vitro* responsiveness in the males is delayed as compared with the females, but no such difference was seen in cultures with PPD. Figure 4 also shows that cells from only 50% of the males finally showed an *in vitro* response to BES.

Table 4. Lymphocyte transformation. Peripheral blood leucocytes from female guinea-pigs immunized with ES in FCA.

Weeks after immunization	Cultures with PPD S.R.	Mean ±SD	Cultures with BES S.R.	Mean ±SD
	32·6		5·3	
3	22·7		5·7	
	11·9	15·8	10·7	5·7
	4·0	±11·4	3·8	±3·0
	7·6		2·9	
	13·8		6·4	
4–5	6·9		5·0	
	28·2	13·7	5·1	7·9
	8·7	±8·5	13·0	±3·5
	10·7		10·0	

The animals at 4–5 weeks had been skin tested three times previously with PPD.

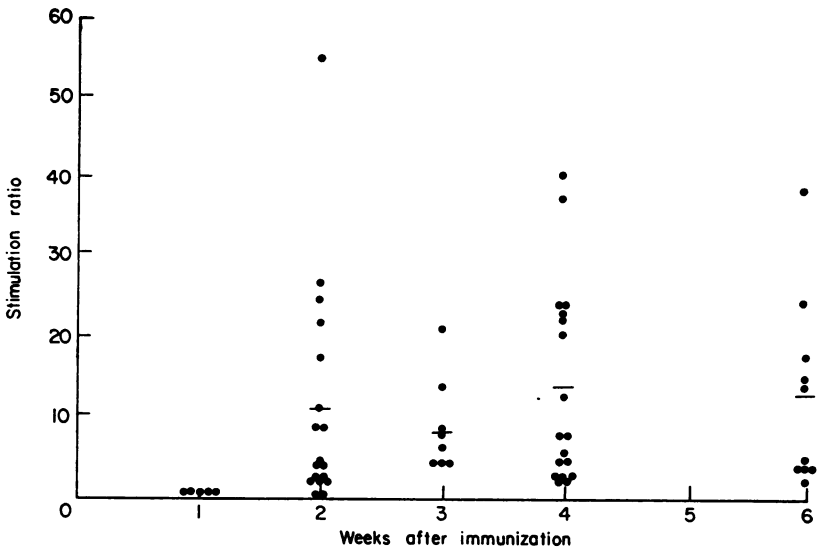

Figure 3. Lymphocyte transformation. Cultures with PPD. Peripheral blood leucocytes from male guinea-pigs immunized with ES in FCA. Each dot represents the S.R. from one animal.

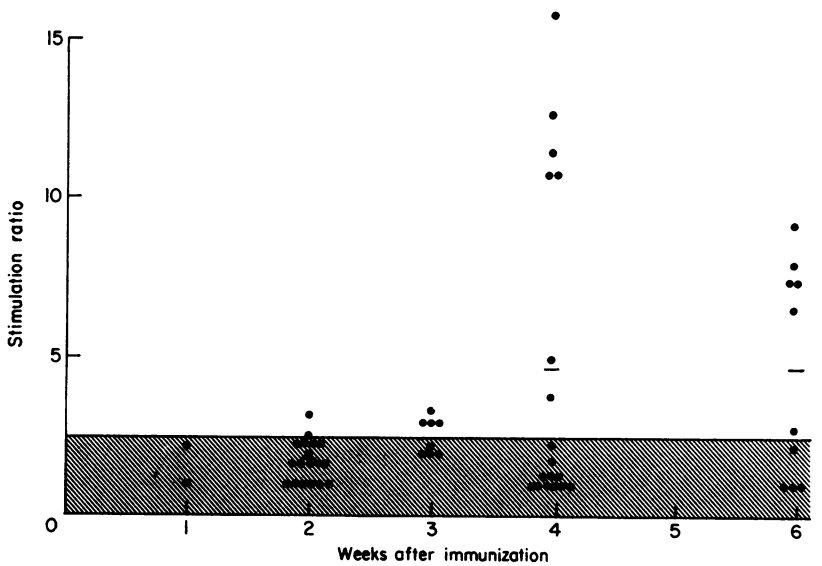

Figure 4. Lymphocyte transformation. Cultures with BES. Same guinea-pigs as in Fig. 3. Hatched area represents level of non-specific stimulation.

We wondered if the *in vitro* response to PPD developed particularly early. Therefore in Table 5 is shown the result of culturing blood leucocytes from male guinea-pigs immunized with OA in FCA. Nearly all the cultures with OA and 50% of cultures with PPD, show a significant uptake of [^{3}H]-thymidine (S.R. > 2·0).

Histological studies

Characteristics of the orchitis developing after immunization with ES in FCA have been described previously (Muir *et al.*, 1976). All the testes described in Table 2 were examined and all showed aspermatogenesis which was most severe in the group immunized with 10 mg TS.

The adrenals and ovaries from four female guinea-pigs immunized with ES in FCA showed no evidence of pathology.

DISCUSSION

Reduced skin responsiveness was shown in male as compared with female guinea-pigs immunized with the aspermatogenic antigens (ES, TS) in FCA, at 2 weeks after immunization (Figs 1 and 2). We had shown previously, that in males immunized with ES in FCA, the skin response was minimal at this time (Muir *et al.*, 1976). As compared with the skin response induced by 10 mg TS, 20 mg caused significant unresponsiveness in females and a profound unresponsiveness in males (Table 1).

Table 5. Lymphocyte transformation. Leucocytes from male guinea-pigs 2 weeks after immunization with 5μg OA in FCA

	Cultures with PPD 50 μg/ml	Cultures with OA 100 μg/ml	10 μg/ml
S.R. per guinea-pig	4·5	6·0	6·1
	2·5	2·3	2·4
	8·9	5·8	7·0
	2·5	1·9	1·7
	0·8	1·5	2·4
	1·3	2·3	2·1
	0·8	2·9	5·7
	1·0	3·4	10·9
Mean S.R. ± S.D.	2·8 ±2·8	3·2 ±1·7	4·8 ±3·2

The degree of aspermatogenesis also varied with the quantity of antigen used for immunization. 10 mg TS, which induced the largest skin response in females, caused most aspermatogenesis in the males. However, if the reduced skin response in males is caused by the aspermatogenesis, as suggested above, it is surprising that those animals with the less severe testis lesion (immunized with 20 mg TS) should show almost no skin response.

The males also showed corresponding anergy in the *in vitro* studies. At 2 weeks, the PEC showed non-specific anergy (to PPD); the blood leucocytes only specific unresponsiveness (to BES). Unresponsiveness of PEC to BES was not excluded. However, the clear delay in development of the response of blood leucocytes to BES (as compared with the response in females), suggests that at two weeks after immunization, cells sensitized to BES either were not present in blood, or were unable to respond. As male guinea-pigs immunized with OA in FCA showed significant *in vitro* responses to both OA and PPD at 2 weeks and the females showed *in vitro* responses to both BES and PPD, unresponsiveness *in vitro* was specific to these guinea-pigs developing aspermatogenesis, that is, the autoimmunity modified the efferent response.

The question arises first, as to whether these studies establish that the reduced skin response is also caused by the aspermatogenesis and second, by what mechanisms the efferent responses might be reduced. Although orchidectomy might elucidate the effect of the testis lesion upon the skin response, this was not practical. Therefore the validity of the females as controls must be examined.

The females used for these studies remained healthy throughout and showed no evidence of autoimmunity. Their skin and *in vitro* responses to BES resembled those to a standard protein antigen, whereas in the males, skin and *in vitro* responses differed both from those shown by females and from those shown by male guinea-pigs immunized with standard protein antigens. As there is no evidence that the sex of an animal influences the immune response to an antigen injected with FCA, it must be concluded that the suppressed efferent responses were specific to animals developing aspermatogenesis and therefore that the females served as valid controls.

After immunization with ES in FCA, males showed a significant skin response to BES at 1 week, but reduced skin reactivity at 2 weeks. Non-

specific reactivity to PPD which was initially suppressed, had become normal by the time the non-specific response was minimal (Muir *et al.*, 1976). Such findings were paralleled by Kantor (1976), who desensitized guinea-pigs by daily injections of protein antigens. Figure 2 confirms that anergy was confined to the delayed hypersensitivity response, the Arthus reaction being unaffected. Both specific and non-specific unresponsiveness of PEC has been described after desensitization (Schlossman, Leven, Rocklin & David, 1971; Poulter & Turk, 1976), when lymphocytes reactive to the specific antigen are trapped within lymph nodes. However, if such lymphocytes are trapped, they will not be circulating in the blood.

As the results of these and the previous study provide many parallels with experimental studies of desensitization, it is suggested that this mechanism causes the reduced reactivity exhibited by the male guinea-pigs immunized with aspermatogenic antigens. Therefore, it is pertinent to look for an event *in vivo*, analogous to the desensitizing injections. Toullet & Voisin (1974; 1976) showed that during the development of experimental autoimmune orchitis in guinea-pigs, sperm antigens are released into the circulation. When aspermatogenesis develops after immunizing guinea-pigs with ES or TS in FCA, the seminiferous tubules become depleted of their contents, that is, sperm antigens. As such loss of tubular contents was apparent at a time when there was little or no cellular infiltration (Muir *et al.*, 1976), it is probable that at least some of the sperm antigens circulated in the blood, thereby acting to desensitize the animal. Support for this is provided by the observation, that after immunization with ES in FCA, the skin response to BES became maximal only when the seminiferous tubules were almost empty. The profound anergy induced in the male guinea-pigs immunized with 20 mg TS therefore, might be caused by the additional sperm antigens released from the damaged testis.

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