Cell-mediated and humoral immune responses to aspermatogenic antigen in experimental allergic orchitis in the guinea-pig

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Summary. Guinea-pigs were immunized with a defined and highly potent aspermatogenic antigen, G75m, and the occurrence of orchitis was correlated with (1) cell-mediated immune responses to G75m, determined by lymph node cell proliferation and by secretion of macrophage migration inhibitory factor (MIF) by peritoneal exudate cells, and (2) humoral antibodies to G75m and to cell surface antigens of guinea-pig testicular cells, by radioimmunometric assays. A consistent temporal relationship between cell-mediated immune responses and disease was found: lymph node cell proliferation was positive by Day 4, followed 3 days later by maximum secretion of MIF, and orchitis lesions were manifest on Day 10. In contrast, maximal IgG antibodies to G75m or to the surface antigens of spermatozoa/testicular cells were detected at a time when cell-mediated immune responses and active testicular lesions had subsided. In individual animals, lymph node cell proliferation increased with severity of orchitis, while MIF secretion by peritoneal cells increased with orchitis only late in the disease. Early in disease, MIF response showed a negative correlation with orchitis. Moreover, peritoneal injection of oil reduced the incidence of early lymph node cell proliferative responses, and delayed the onset of testicular disease. These findings are consistent with competition between different inflammatory sites for recently antigen-activated T lymphocytes. We conclude that (1) the development of orchitis correlates with cell-mediated immune responses to purified aspermatogenic antigens but not with IgG antibody responses, and (2) when the same animal is used to assess different aspects of cellular immunity and autoimmune disease, one study may significantly influence the other.

Introduction

Since the first descriptions of experimental allergic orchitis (Voisin, Delaunay & Barber, 1951; Freund, Lipton & Thompson, 1953), studies of the immunopathology and pathogenesis of this disease have been based on crude extracts from guinea-pig testis or spermatozoa. Since sperm/testis autoantigens may not be aspermatogenic (i.e. may not induce allergic orchitis) (Hagopian, Limjuco, Jackson, Carlo & Eylar, 1976), investigation of correlation between allergic orchitis and the immune responses to crude tissue extracts may yield misleading or confusing results. Several aspermatogenic proteins and glycoproteins from guinea-pig testis have been isolated and characterized (Jackson, Hagopian, Carlo, Limjuco & Eylar, 1975, 1976; Hagopian, Jackson, Carlo, Limjuco & Eylar, 1975; Hagopian *et al.*, 1976). However, information on the development and progression of testicular lesions induced by these antigens is sparse (Hagopian *et al.*, 1975; Carlo,

Hagopian, Jackson, Limjuco & Eylar, 1976). We have purified two aspermatogenic proteins, one from guinea-pig spermatozoa, named AP2 (Teuscher, Wild & Tung, 1983a), and the other from guinea-pig testis, AP3 (Teuscher *et al.*, 1983b). The physicochemical characteristics of these antigens are different from those of aspermatogenic antigens already described: AP2 is an acidic protein of M_r 9500, whereas AP3 is a basic protein (M_r 12 500) that retains aspermatogenic activity after reduction and alkylation. Since the yields of these antigens are low, it would be impractical and excessively costly to study in detail allergic orchitis induced by them.

During the purification of AP3, a preparation of proteins/glycoproteins of M_r 12 000–30 000, known as G75m, was found to be highly active in inducing allergic orchitis. We have therefore studied the histopathology of experimental allergic orchitis induced by G75m, and searched for relationships between the disease process and humoral and cell-mediated immune responses to G75m.

Materials and Methods

Animals

Adult male Hartley non-inbred guinea-pigs with a mean $\pm$ s.d. body weight of 647 ± 108 g ($N = 117$) were purchased from Camm Research Institute, Inc. (Wayne, NJ).

Isolation of aspermatogenic antigen, G75m

The purification steps were a modification of the method for isolation of the aspermatogenic antigen AP1 (Jackson *et al.*, 1975). Two batches of G75m antigen were used in our study, called pool A and pool B. Pool B antigen was prepared according to Teuscher *et al.* (1983b) and included the following steps: (1) homogenization of the testes, (2) lipid extraction, (3) acid extraction, (4) removal of materials that precipitate at pH 7, (5) ammonium sulphate precipitation, (6) trichloroacetic acid precipitation and (7) Sephadex G75 column chromatography. In the preparation of pool A antigen, step 4 was omitted and, in step 5, ammonium sulphate was not dialysed. Antigen concentrations were assessed by absorbance at 280 nm, assuming $1 \text{ mg/ml} = 0.60$. Both pools consistently induced orchitis at 2–10 μg per guinea-pig. The freeze-dried preparations of G75m were reconstituted with saline (9 g NaCl/l) to about 4 mg/ml, filter-sterilized, and stored at 4°C.

Immunizations

One volume of G75m was homogenized into 1 volume of Freund's complete adjuvant containing 0.5 mg *Mycobacterium tuberculosis* H37Ra/ml (DIFCO, Detroit, MI). Each guinea-pig was immunized with 50 μg G75m in 0.2 ml, distributed among the four footpads. Control guinea-pigs were either unimmunized or immunized with Freund's complete adjuvant.

Preparation of lymph node cells

Guinea-pigs were exsanguinated by cardiac puncture under light ether anaesthesia. Subsequent work was done aseptically; materials and reagents were sterile, and kept on ice unless stated otherwise. Popliteal, superficial inguinal, axillary and subscapular lymph nodes were removed and placed in RPMI 1640 medium (GIBCO, Santa Clara, CA) with 10 mM-Hepes, 10 units heparin/ml and 10 μg DNase/ml (Sigma, St Louis, MO), called Medium RHHD. Lymph nodes were minced in Medium RHHD, pressed through a 60-mesh stainless-steel sieve, filtered through nylon cloth (Nitex No. 110, TETCO, Inc., Elmsford, NY) and centrifuged at 300 g for 10 min at 10°C. The lymph node cells were resuspended in 80 ml Medium RHHD. To separate live from dead cells, each

20 ml was layered onto 10 ml hypaque-ficoll (Parish, Kirov, Bown & Blanden, 1974), without sodium azide at about 25°C, and centrifuged at 1600 *g* for 15 min at 20–25°C. The cells from the interface were washed twice with Medium RPMI 1640 + 10 mM-Hepes before they were resuspended in the culture medium to 5×10^6 viable cells/ml. The culture medium was Medium RPMI 1640 with 10 mM-Hepes, 100 µg gentamycin/ml, and 10% heated-inactivated normal guinea-pig serum (Pel Freez, Rogers, AK).

Preparation of peritoneal exudate cells

These cells were harvested 3 days after stimulation with 20 ml sterile mineral oil (Humco Lab, Texarkana, TX). After exsanguination and lavage of the peritoneal cavity, the cells were obtained by centrifugation at 500 *g* for 10 min at 10°C, pooled and resuspended with 10–15 ml lysing solution (Roos & Loos, 1970) and kept on ice for exactly 5 min with periodic mixing. Peritoneal exudate cells were washed twice with Eagle's minimum essential medium (MEM) with Hanks' salts and glutamine, and resuspended in culture medium to 65×10^6 /ml. The culture medium was MEM with Earle's salts and glutamine (Flow Laboratories, Rockville, MD), 10 mM-Hepes, 100 µg gentamycin/ml, and 15% heat-inactivated serum from normal guinea-pigs; this serum pool had had lipids and aggregates removed by centrifugation. Only cells with a viability >90% were used. Differential counts of peritoneal exudate cells stained with Giemsa consisted of 80–85% large mononuclear cells, 5–10% smaller mononuclear cells, and neutrophils.

In-vitro assays

Lymph node cell. Antigen-initiated incorporation of [³H]thymidine by the lymph node cell was set up in microtitre plates (Flow Laboratories, Rockville, MD). Each well contained 100 µl of a dilution of G75m in culture medium and 100 µl of lymph node cells (5×10^5). The plates were incubated for 24 h at 37°C in a humidified atmosphere of 5–6% CO₂ in air. Subsequently, the lymph node cells were pulsed with 0.5 µCi [³H]thymidine (sp. act. 2 Ci/mmol, Amersham Corp., Arlington Hts, IL), cultured for 24 h, and collected with a MASH II cell harvester (Microbiological Associates, Walkersville, MD) onto glass-fibre filters. Radioactivity was measured with a Packard 2420 beta-spectrometer using Scint-A fluid (Packard, Downers Grove, IL). The lymph node cell stimulation index (SI) was calculated as net c.p.m. experimental/net c.p.m. control, i.e. (lymph node cells + test reagent)/(lymph node cells + culture medium only). For a given guinea-pig, the lymph node cell assay was considered positive when the SI was >2.0 at any of the concentrations tested. In addition to G75m at 12.5–200 µg/ml final concentration, test reagents were preservative-free tuberculin-purified protein derivative (PPD-CT68 Lot A21: Connaugh Laboratories, Swiftwater, PA) at 6.25–100 µg/ml, and phyto-haemagglutinin M (10 mg/ml: Difco; Control No. 664780) at 1/2560 to 1/160 dilutions.

Peritoneal exudate cells. The direct assay for macrophage migration inhibitory factor (MIF) with peritoneal exudate cells was done in glass capillary tubes placed in leukocyte migration plates (Leu, Woodson & Whitley, 1977). Four control wells (8 capillaries) filled with culture medium only were used, 2 at the start and 2 at the end of the set-up for a guinea-pig; each concentration of the test reagent occupied 2 wells. G75m was tested at 10–200 µg/ml, and purified protein derivatives at 10 and 50 µg/ml. The 24-h migration patterns were projected to constant magnification, traced, and measured by planimetry. The cell migration index was calculated as 100 (mean area of migration in experimental wells/mean area in control wells). For each guinea-pig, the experimental areas were compared to its own control areas by Student's *t* test. Macrophage migration was considered to be inhibited, or enhanced, significantly when the *P* value was <0.05.

Assay of serum antibody against surface autoantigens on residual bodies, late spermatids and spermatozoa. The antibodies were measured as described by Han & Tung (1979).

Solid-phase radioimmunoassay for antibody to G75m

Flat bottom, 96-well polystyrene microtitre plates (Dynatech Laboratories, Alexandria, VA) were coated with G75m, 25 µg in 0.5 ml per well. After drying by overnight storage at room temperature, the wells were washed three times with phosphate-buffered saline (PBS) containing 0.3% gelatin and 0.03% EDTA (RIA buffer). The test serum, 50 µl diluted 1/20 in PBS, was added to each well and incubated at room temperature for 3 h. Each serum was tested in quadruplicate. The serum samples were removed, and the wells were washed 3 times with 150 µl RIA buffer. After a fourth wash with 150 µl 1% IgG-free bovine serum albumin (BSA) and a 5-min incubation with the same buffer, 0.02 µg ¹²⁵I-labelled protein A in 50 µl (about 100 000 c.p.m.) was added to each well and incubated for 3 h. The wells were washed 3 times with 150 µl 1% IgG-free BSA, and were dried. The radioactivity of the well bottoms was determined in a gamma-spectrometer. A standard anti-G75m antiserum from guinea-pigs hyperimmunized with G75m produced a linear dose-response curve in the dilution range of 1/64 to 1/2, with a well-bound radioactivity of 2000–10 000 c.p.m. Normal guinea-pig serum samples consistently showed anti-G75m activity of 1000–2000 c.p.m. For the experimental sera, anti-G75m activity was considered to be positive when the well-bound c.p.m. was >3000, and highly positive when >5000 c.p.m.

Histopathology of the testes

The testes were fixed in Zenker's fluid, embedded in paraffin wax, and 5 µm-thick semisequential sections were stained with periodic acid-Schiff's-haematoxylin (Putt, 1972). The absence or presence and type of pathology was assessed for the seminiferous tubules, rete testis, ductuli efferentes, and cauda epididymidis. A guinea-pig was considered to have allergic orchitis when inflammatory cellular infiltrates were found in one or more of the areas mentioned. The severity of the orchitis was assessed by the extent of cross-sectional areas infiltrated, the degree of infiltration, the presence (or absence) and extent of fibrosis, and the extent of aspermatogenesis.

Experimental groups and statistical analyses

A first group of guinea-pigs was immunized and tested with G75m pool A. Of these, 8 animals were killed 4 days after immunization, 14 at 7 days, 12 at 10 days, 14 at 14 days, 6 at 19 days and 4 at 29 days. Lymph node and peritoneal exudate cells were taken from 12 guinea-pigs, while the others were used for one or the other assay, since we noticed that i.p. injection of oil appeared to affect orchitis and the lymph node cell assay. To assess the effects of i.p. oil injection and to test for reproducibility of the immunogen, a second group of guinea-pigs was immunized with G75m pool B. This group comprised 8 animals killed 7 days after immunization (4 lymph node cell + peritoneal exudate cells), 24 at 10 days (12 for histopathology only, 8 for lymph node cell, 4 for peritoneal exudate cells) and 8 at 19 days (4 lymph node cells, 4 peritoneal exudate cells).

All statistical tests were done at 95% confidence level. For the analyses of incidences, guinea-pigs from different groups (+oil and -oil, pool A- and pool B-immunized) within a time period were combined when the χ^2 test for homogeneity permitted it. Incidences were analysed by Fisher's Exact test, and by G and χ^2 tests for independence when appropriate. Lymph node cell stimulation and peritoneal exudate cell migration indices were subjected to Analysis of Variance followed by Duncan's Multiple Range tests. The relationship between orchitis and the in-vitro assays was analysed by Kendall's Paired Rank Correlation (Campbell, 1974).

Results

Histopathology

The peak incidence of animals with histopathology occurred at Day 14 in guinea-pigs immunized with pool A of G75m antigen, and at Day 10 with pool B antigen (Table 1). Before and on Day 14, orchitis occurred without aspermatogenesis in most guinea-pigs (33/46, 72%, Table 2).

Table 1. Development of epididymo-orchitis, and of immune responses to G75m antigens in guinea-pigs immunized with G75m in Freund's complete adjuvant

Studies	G75m antigen	Days after immunization					
		4	7	10	14	19	29
1. Histopathology	Pool A	1/8 (13%)	4/14 (29%)	4/12 (33%)	12/14 (86%)	5/6 (83%)	4/4 (100%)
	Pool B	*	3/8 (38%)	22/24 (92%)	*	8/8 (100%)	*
2. MIF production by PEC	Pool A	0/4 100 ^a	1/4 (25%) 46	5/6 (83%) 47 ± 6.8	5/7 (71%) 33 ± 2.4	3/4 (75%) 53 ± 5.0	0/2
	Pool B	*	4/4 (100%) 46 ± 13	4/4 (100%) 18 ± 4.1	*	4/4 (100%) 35 ± 15	*
3. LNC proliferative response	Pool A	1/4 (25%) 2.1 ^b	6/9 (67%) 6.6 ± 1.6	7/8 (88%) 5.4 ± 0.9	4/6 (67%) 3.5 ± 0.7	3/4 (75%) 2.5 ± 0.2	0/2
	Pool B	*	5/7 (71%) 5.6 ± 0.6	8/8 (100%) 5.7 ± 0.8	*	4/4 (100%) 3.4 (0.5)	*
4. Anti-TSDA antibody	Pool A	2/6 (33%) 11 ± 2 ^c	2/4 (14%) 11 ± 1	3/12 (25%) 12 ± 1.5	3/12 (25%) 10 ± 0.4	2/6 (33%) 12 ± 4.8	1/4 (25%) 9.2
	Pool B	*	3/7 (43%) 16 ± 5.9	9/14 (64%) 18 ± 2.6	*	7/8 (88%) 15 ± 3.4	*
5. Anti-G75m antibody	Pool A	0/6 —	0/14 —	2/12 (17%) 3016 ^d	4/12 (33%) 3124	3/6 (50%) 3842	4/4 (100%) 5223
	Pool B	*	0/7 —	4/14 (29%) 3503	*	5/8 (63%) 5416	*

MIF, macrophage migration inhibitory factor; PEC, peritoneal exudate cells; LNC, lymph node cells; TSDA, testicular cell/sperm differentiation autoantigens.

Values for the migration index^(a), stimulation index^(b), and anti-TSDA antibody level^(c) (µg/ml serum) are mean ± s.d.

^(d) means c.p.m. values for guinea-pigs with positive anti-G75m antibody responses (normal range = 1000–2000 c.p.m.).

* Not studied.

Table 2. Distribution of testicular histopathology in guinea-pigs immunized with G75m antigens (pool A or B)

	Days after immunization							
	4*	7		10		14*	19 and 29	
	A	A	B	A	B	A	A	B
Aspermatogenesis	0	0	0	1	1	0	0	0
Inflammation of seminiferous tubules only	0	1	0	1	4	0	1	0
Inflammation in rete/ductus efferentes only	1	2	2	0	1	2	0	0
Inflammation in cauda epididymidis only	0	0	0	0	0	0	0	0
Inflammation in seminiferous tubules, rete and ductus efferentes	0	1	0	1	6	1	0	0
Inflammation in rete/ductus efferentes and cauda epididymidis	0	0	1	0	0	0	0	0
Inflammation in seminiferous tubules, rete and ductus efferentes and cauda epididymidis	0	0	0	2	10	9	0	0
Fibrotic ductus efferentes and rete testis	0	0	0	0	1	0	8	8

* Pool B antigen was not studied on these days.

Values are for no. of guinea-pigs; see text for no. of animals in each group.

Aspermatogenesis alone occurred in only 2 of the 65 animals in the whole study. Early orchitis was found mainly in the rete testis and ductuli efferentes, although the seminiferous tubules and the cauda epididymidis were also involved by Day 10. However, in 6 of the 46 guinea-pigs (up to Day 14) orchitis was exclusively in the seminiferous tubules. Fibrosis occurred later after immunization.

Intraperitoneal injection of mineral oil at Day 7 reduced the incidence of orchitis at Day 10 in guinea-pigs immunized with pool A antigen ($P < 0.05$), but the frequency of orchitis on Day 7, 14 or 19 was the same regardless of whether the guinea-pigs were injected with oil 3 days earlier (data not shown).

Immune responses

Proliferative response of lymph node cells to G75m. The lymph node cells from unimmunized or adjuvant-immunized guinea-pigs were not stimulated by G75m to incorporate significant amounts of [^3H]thymidine; the effects of immunization with G75m CFA are summarized in Table 1 (Study 3). The responses to G75m pool A and pool B antigens were similar. The incidence and magnitude were maximal by Day 7, and the magnitude declined from Day 10. The 2 animals studied on Day 29 were unresponsive.

Intraperitoneal injection of mineral oil at Day 4 significantly reduced the response of lymph node cells on Day 7 ($P = 0.04$) (data not shown).

Inhibition of peritoneal macrophage migration. The migration of peritoneal macrophages from unimmunized or adjuvant-immunized guinea-pigs was unaffected in the presence of 10–50 μg G75m but was enhanced at higher doses. The incidence of response to pool A and pool B G75m was maximal at Day 10 and Day 7, respectively, while the magnitudes peaked at Days 14 and 10, and declined thereafter (Table 1, Study 2). The 2 guinea-pigs studied on Day 29 were unresponsive.

Anti-testicular cell surface antibody response. Our previous study established that the mean $\pm$ s.d. level of antibody to testicular cell surfaces in normal adult male Hartley guinea-pigs was $3 \pm 2 \mu\text{g/ml}$ (Tung, Bryson, Goldberg & Han, 1979). In the present study, the mean $\pm$ 2 s.d. value was exceeded by 13 of 54 (24%) guinea-pigs immunized with pool A antigen, and 19 of 29 (66%) animals immunized with pool B antigen. Furthermore, the incidence of response of the latter animals increased significantly with time (Table 1, Study 4). However, the magnitude of antibody response did not differ between the two groups.

Anti-G75m antibody response. Guinea-pigs immunized with Freund's complete adjuvant did not develop anti-G75m antibody. Although a few of the G75m-immunized animals had low levels of serum antibodies between Days 10 and 14, a higher proportion of animals studied on Days 19 and 29 were positive, and the antibody levels were higher (Table 1, Study 5). There was no association between positive responses for antigens to G75m and testicular cell surfaces; of 97 serum samples tested, 23% were positive only for the testicular cell surface antibody, while 12% were positive only for the G75m antibody. Consistently, the onset of cell-mediated immune responses preceded that of humoral antibody responses to G75m. Maximum titres of anti-G75m antibodies were found in guinea-pigs in which the cellular immune response to G75m had subsided.

Relationship of the immune responses to orchitis

There was a consistent temporal relationship between the incidence and magnitude of cellular immune responses to G75m antigen and the development of orchitis. In guinea-pigs immunized

with pool A antigen, the peak proliferative response of lymph node cells was on Day 7, followed by peak incidences of peritoneal macrophage response on Day 10 and of orchitis on Day 14, when the magnitude of peritoneal macrophage response also peaked. The temporal relation between cell-mediated immune responses and orchitis in guinea-pigs immunized with pool B antigen was similar, except that each of the indicators had an earlier onset (Table 1).

Table 3 summarizes the paired incidences of orchitis and cell-mediated immune responses to G75m antigen in individual guinea-pigs. Animals immunized with pool A or pool B antigens were studied together. Guinea-pigs without orchitis but exhibiting a positive or a negative proliferative response of lymph node cells belonged exclusively to animals studied before Day 14. Guinea-pigs with orchitis and a negative lymph node cell response clustered in the period beyond Day 10. Although a similar pattern pertained to the orchitis/migration inhibition factor, several guinea-pigs studied early after immunization had orchitis in the absence of MIF responsiveness (Table 3, last column).

Table 3. Paired incidences for orchitis and lymph node cell (LNC) proliferative assay or macrophage migration inhibitory factor (MIF) assay as a function of time after immunization

Day	G75m pool	No. of animals	Orchitis/LNC proliferative assay				No. of animals	Orchitis/MIF assay			
			-/-	-/+	+/+	+/-		-/-	-/+	+/+	+/-
4	A	4	1	3			4	3			1
7	A	9	3	3	3		4	2	1		1
	B	7	1	4	2		4		3	1	
10	A	8	2	3	3		6		5		1
	B	8		1	7		4		1	3	
14	A	6			4	2	7	1		5	1
19	A	4			3	1	4	1		3	
	B	4			4		4			4	
29	A	2				2	2				2
Total (%)		52	7 (13%)	14 (27%)	26 (50%)	5 (10%)	39	7 (18%)	10 (26%)	16 (41%)	6 (15%)

The relationships between severity of orchitis and magnitude of cellular immune responses to G75m antigen were analysed in guinea-pigs that were positive in both variables. Irrespective of which time periods were analysed, alone or together (Days 7 and 10; Days 10 and 14; Days 14 and 19; Day 19), there was a positive, although not always significant, correlation between orchitis and proliferative responses of lymph node cells. The relationship between increasing severity of orchitis and increasing responsiveness of peritoneal macrophages was such that a negative correlation characterized the period Days 7 to 14 ($\tau = -0.5$, $S = -21$, $n = 9$), whereas a positive correlation ($\tau = +0.81$, $S = +17$, $n = 7$) existed for guinea-pigs studied after Day 14.

Discussion

Several findings prompted this study of cell-mediated immune response and humoral antibody response to sperm antigens as possible correlates to experimental allergic orchitis. Vasectomy in the rabbit led to immune complex orchitis (Bigazzi, Kosuda, Hsu & Andres, 1976), with lesions resembling those that are transferrable by guinea-pig lymphoid cells to syngeneic recipients (Tung, 1978). Although orchitis has not been detected in men after vasectomy, this possibility has not been ruled out. Likewise, testicular autoimmunity has not been excluded as a cause of the high frequency of infertility observed in men following vasovasostomy (Linnet & Hjort, 1980). It is, therefore, important to develop an in-vitro test indicative of testicular autoimmune disease. A study of testis biopsies from infertile men revealed evidence of immunological mechanisms in the form of

immune complexes (Solomon, Saremaslani, Jakob & Hedinger, 1982). Since experimental autoimmune orchitis appears to be a disease caused mainly by cell-mediated mechanisms, it is important to study infertile men, and perhaps women, for cell-mediated immune responses to sperm antigens.

It is known that spermatozoa and testis tissue possess numerous autoantigens, not all of which are capable of inducing allergic orchitis (Hagopian *et al.*, 1976). Correlative studies between cell-mediated immune responses to spermatozoa and orchitis are only meaningful if antigen(s) relevant to induction of the disease are used. The potent aspermatogenic protein, AP3, is the only detectable aspermatogenic antigen in an extracted, delipidated preparation of guinea-pig testicular proteins with a molecular weight between 15 000 and 30 000 (G75m) (Teuscher *et al.*, 1983b). In this study, we used two in-vitro methods to evaluate the cell-mediated immune responses to G75m of guinea-pigs immunized with G75m, and examined their relationship to humoral antibody responses to sperm antigens, and to the development of orchitis. The results demonstrated a convincing temporal pattern of cell-mediated immune responses in relation to orchitis development. Antigen-induced specific proliferation of lymph node cells was detected as early as 4 days after immunization. This was followed 3 days later by the maximum lymph node cell response, which was followed by maximum antigen-induced secretion of migratory inhibitory factor by peritoneal exudate cells when lesions of orchitis became manifest. This sequence of immunopathological events was reproducible even when two different preparations of G75m antigens were used. While the three biological events occurred earlier in guinea-pigs immunized with pool B antigen, the order in which these events were manifest remained the same. These findings indicate that G75m-reactive lymphocytes were present within the regional lymph nodes within 4 days after induction of orchitis. These cells soon circulated and could be attracted to the inflammatory reaction induced by oil in the peritoneal cavity.

The nature of the relationship between orchitis and cell-mediated immune response to G75m in individual animals was dissimilar for the peritoneal exudate and lymph node cells. The magnitude of the proliferative response of lymph node cells showed positive correlation with severity of active testicular disease. In contrast, the peritoneal exudate cells obtained on or before Day 14 produced less migration inhibitory factor when the orchitis was more severe.

In contrast to cell-mediated immune responses, humoral antibodies to G75m or to cell surface antigens of testicular cells bore no correlative relationship to the presence of orchitis. Thus, peak antibody levels were detected at a time when cell-mediated immune responses had subsided, and when the active phase of the disease had waned. The data from this study support the suggestion that orchitis in the guinea-pig is an immunological disease induced mainly by cell-mediated immune mechanisms. Additional evidence for this derives from a number of observations. First, the histopathology of orchitis is that of cell-mediated immunological injury (Waksman, 1959; Tung, Unanue & Dixon, 1970). Second, the orchitic lesions can be transferred locally (Tung *et al.*, 1971a; Kantor & Dixon, 1972) and systemically (Carlo *et al.*, 1976) with lymphoid cells, or with enriched T lymphocytes (Tung, Leong & McCarty, 1977). In contrast, focal disease can be transferred with difficulty by immune sera, and recipients had to receive Freund's complete adjuvant before the disease is induced (Tung *et al.*, 1971b; Toullet & Voisin, 1976). Third, murine orchitis cannot be induced in nude mice, but can be induced in nude mice reconstituted with thymic cells from their +/nu littermates (Bernard, Mitchell, Leydon & Bargerbos, 1978). Also, studies on the antigen AP3 have shown that it retains its aspermatogenic property after reduction and alkylation, suggesting a role for amino acid sequential antigen determinant, and not conformation determinant, in disease induction (Teuscher *et al.*, 1983b). Gell & Benacerraf (1959) have provided evidence that sequence antigenic determinants elicit mainly cell-mediated immune responses.

There have been several studies on the cell-mediated immune responses to sperm antigens in guinea-pigs with orchitis (Freund, Thompson & Lipton, 1955; Baum, Baughton, Mongar & Schild, 1961; Brown, Glynn & Holborow, 1967; Voisin & Toullet, 1969; Mazzolli, 1971; Marcus, Nebel, Soffer, Stahl & Toullet, 1975; Muir & Turk, 1979; Hojo, Hiramane & Ishitaki, 1980). Taken

together, these studies provide evidence for an overall association between positive cell-mediated immune responses and orchitis. Hojo *et al.* (1980) studied the response of lymph node cells in guinea-pigs to an antigenic preparation that probably contained G75m and detected an initial peak of cell-mediated immune response, followed by a second peak after active testicular disease had completely subsided. In our study and that by Hojo *et al.* (1980) decline of active orchitis was associated with diminution of cell-mediated immune responses. Whether this results from the activity of suppressor cells or suppressor factors needs further investigation. It is possible that the second peak of cell-mediated immune response detected by Hojo *et al.* (1980) represents immune response to endogenous antigens released from the diseased testes. Earlier studies (Brown *et al.*, 1967; Voisin, 1982) based on different antigenic preparations have led to the conclusion that both cell-mediated immunity and humoral antibodies might be involved in the pathogenesis of allergic orchitis.

The findings in this study of (1) the effect of oil-induced peritoneal exudate on the cell-mediated immune response of lymph node cells and on the time of onset of orchitis, and (2) the negative correlation between orchitis and response to migration factors early after disease induction are consistent with preferential recruitment of recently activated T lymphocytes into inflammatory sites (Koster, McGregor & Mackaness, 1971; Asherson, Allwood & Mayhew, 1973; McGregor & Logie, 1974) and competition for these cells by different inflammatory loci. Treatment with oil may therefore reduce the number of antigen-reactive cells in the lymph nodes, and in the circulating pool capable of orchitis induction. Care should therefore be taken when the same animal is used to assess different aspects of cell-mediated immune responses, inasmuch as the experimental manipulation required for one study may significantly influence the other, including manifestation of disease.

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