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Experimental Allergic Encephalomyelitis in Hamsters[1] (37769)

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In ongoing studies of the immunobiology of experimental allergic encephalomyelitis (EAE), we found the Syrian hamster (*Mesocricetus auratus*) comparatively insusceptible to this disease [cited in conference discussion (1)]. Our preliminary findings were surprising on two counts. First of all, Tal, Laufer and Behar (2) have reported that EAE can be induced in hamsters with relative ease. Second, this species of animal has proved useful in studies of other immunologic systems, e.g., transplantation immunology (3).

This paper will summarize our evidence that the Syrian hamster has a relatively low order of susceptibility to EAE, in comparison with most other mammalian and avian hosts (4). In addition, our data indicate that the limited capacity of hamsters to develop EAE is not explained by immunosuppressive activity of splenocytes, impaired synthesis of antibody to neuroantigens or gross deficiency of encephalitogenic neuroantigen in hamster central nervous system (CNS) tissue.

Materials and Methods. Animals. Fully mature (at least 8 wk old) "Golden" Syrian hamsters were obtained from Con-Olson Co., Inc., Madison, WI and Lakeview Hamster Colony, Newfield, NJ. Albino or Hartley guinea pigs weighing 500–600 g were purchased from local breeders or Carworth Farms, New York, or the Camm Research Inst., Inc., Wayne, NJ. Rabbits weighing 2 to 4 kg each were procured from local breeders. Lewis strain male rats weighing 125–200 g were secured from Microbiological Associates, Walkersville, MD, or Simonsen Breeders, Gilroy, CA. All animals were maintained on commercial food pellets and water *ad libitum*. In addition, guinea pigs had their diets supplemented with "greens" once or twice weekly.

Sensitizing inocula. Neuroantigen consisted of aseptically removed brain or/and spinal cord, either stored at —20° or used fresh. The CNS tissue was prepared as 10 to 50% homogenates (wet wt) in 0.25% phenol in distilled water. In most cases a 33% homogenate of spinal cord was used. The CNS tissues were homogenized and emulsified in complete Freund's adjuvant (CFA) containing killed, dried *Mycobacterium tuberculosis* H37RV or bacillus Calmette-Guerin (BCG), 4 mg/ml, as previously described (5). Lyophilized hamster CNS was used in preparing some neuroantigen–CFA inocula in order to ascertain the precise amount of neuroantigen injected. Immunization regimens included different routes of sensitization, multiple as well as single injections of neuroantigen–CFA

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emulsions and, in some experiments, supplemental pertussis vaccine to be sure vigorous sensitization to CNS tissue was achieved. Pertussis vaccine (Phase I *Bordetella pertussis*) was bought from Eli Lilly and Co., Indianapolis, IN, or provided as a concentrate containing 200 $\times$ 10^9 bacterial cells/ml by Dr. H. B. Devlin of Parke-Davis and Co., Detroit, MI. In animals sensitized via the footpad route, the Parke-Davis vaccine (0.1 ml) was injected into the dorsum of the same hindfoot that was injected with neuroantigen. In other experiments, the Lilly vaccine was injected intraperitoneally.

Criteria for EAE. The usual clinical neurologic signs which signify occurrence of EAE were as previously described (4, 5). In addition, in experiments utilizing rats, those animals developing only a flaccid or "limp" tail, as described by Levine and Wenk (6), were tabulated as "clinically positive." Brains and spinal cords were obtained from most animals and processed for histopathologic study as described (5). Presence of 1 perivascular mononuclear cellular infiltrate in the 7 sections of CNS routinely examined (cerebrum, mesencephalon, cerebellum-pons and cord) was sufficient to mark an animal as "EAE positive."

Complement-fixation (CF) tests. Either a macro- or micro-CF assay was employed as described previously (7, 8). Two (2) 100% hemolytic units of complement were employed. Optimally diluted standardized ethanolic extracts of lyophilized hamster or rabbit CNS were used as antigen (9).

Results. Efforts to induce EAE in hamsters sensitized to xenogeneic or allogeneic CNS, representing 9 separate experiments, are summarized in Table I. The infrequency of clinical signs of EAE is immediately apparent. The 6 hamsters which developed definite neurologic signs received more than one injection of neuroantigen. The signs first appeared in these hamsters at: 6, 6, 9, 11, 17 and 44 days after the second or third injection of neuroantigen. In 4 animals, clinical neurologic signs were mild and transitory, e.g., grossly ataxic gait for only a few days. Semiflaccid paralysis of the hind legs was observed in only two hamsters.

Lesions of EAE were demonstrable in at least one hamster in each experiment (Table I), except for the 10 animals sensitized to 10% homogenate of hamster brain. A single injection of hamster, rabbit or guinea pig

TABLE I. EAE in Hamsters After Different Regimens of Sensitization to Neuroantigen.

Sensitization regimen[a]		EAE	
CNS tissue	Route and no. inj.	Signs	Lesions[b]
Hamster	Intracut. $\times$ 2	0/10	0/10
Hamster	Subcut. $\times$ 3	0/9	—
Hamster	Footpad $\times$ 1 (+ pertussis vaccine)	0/6	4/6
Rabbit	Intracut. — footpad $\times$ 4	3/10	3/10
Rabbit	Intracut. $\times$ 2 (+ pertussis vaccine)	1/8	4/7
Rabbit	Footpad $\times$ 1 (+ pertussis vaccine)	0/5	1/5
Guinea pig	Intracut. — footpad $\times$ 2	1/9	1/9
Guinea pig	Intracut. — footpad and subcut. $\times$ 2	1/12	5/11
Guinea pig	Footpad $\times$ 1 (+ pertussis vaccine)	0/8	3/8
Guinea pig	Footpad $\times$ 1 (+ pertussis vaccine)	0/5	1/5

[a]CNS tissue inoculum was usually a 33% homogenate of brain and cord or cord, emulsified in adjuvant. Multiple injections given at 12 to 38 day intervals.

[b]Animals usually sacrificed 26 to 28 days after a single injection of neuroantigen or 17 to 47 days after the last injection where multiple injection regimen employed.

neuroantigen into the foot pad, together with pertussis vaccine, induced characteristic cellular infiltrates of EAE (10). Histopathologic changes, however, were more intense and involved multiple areas of the CNS in those animals subjected to multiple injection regimens. Even in these animals the paucity of lesions was impressive, usually only 8 to 10 discrete perivascular cell infiltrates being identified in the sections of brain and spinal cord examined. Myelin stains of sections of spinal cord of selected hamsters with particularly intense cellular infiltrates revealed no appreciable injury to myelin.

Organ-specificity of the CNS lesions in hamsters sensitized to neuroantigen was established by using appropriate control animals. Neither clinical nor histopathologic abnormalities were observed in 16 hamsters sensitized to guinea pig kidney–CFA either with or without supplementary injection of pertussis vaccine.

An important aspect concerning the response of hamsters to the sensitization procedure was the relative lack of inflammatory reaction at the site(s) of injection of neuroantigen–CFA. Even in those animals sensitized via the foot pad route and which also received pertussis vaccine, swelling of the feet was only minimal or moderate, reached a peak about 8 to 10 days following sensitization and then subsided. At no time did the inoculation sites develop into extensive or draining granulomas so commonly seen in other animal species used in EAE studies. Microscopic examination of excised cutaneous inoculation sites did reveal the basic features of granuloma formation. The point we want to emphasize is the relatively small size of the granulomas and the mild character of the inflammatory changes, judging from relative paucity of lymphocytes, histiocytes and multinucleated giant cells.

TABLE II. EAE in Splenectomized and Nonsplenectomized Hamsters.

CNS tissue for sens.[a]	Splenx.[b]	EAE	
		Signs	Lesions[c]
Guinea pig	Yes	0/7	3/7
	No	0/5	1/5
Rabbit	Yes	0/5	2/5
	No	0/5	1/5
Hamster	Yes	0/5	2/5
	No	0/5	4/6

[a]Spinal cord or brain (hamster) in adjuvant injection into hindfoot with pertussis vaccine.

[b]Splenectomy 5, 7 or 9 days before sensitization.

[c]Hamsters sacrificed 28, 33 or 34 days after sensitization; one animal found dead on Day 21.

The effect of splenectomy on the susceptibility of hamsters to EAE is shown in Table II. The splenectomy experiments were prompted by our previous finding that removal of the spleen facilitates cellular transfer of EAE in Wistar rats (11). We were also motivated by the report of Okumura and Tada (12) that the spleen may exert an immunosuppressive influence on antibody synthesis, splenectomy resulting in enhanced levels of circulating antibody following antigenic stimulation. None of the hamsters developed clinical signs of EAE, irrespective of whether or not their spleens were removed. No appreciable difference was observed in the occurrence of lesions of EAE among splenectomized as opposed to nonsplenectomized hamsters.

TABLE III. Complement Fixing (CF) Brain Antibodies in Hamsters Sensitized to CNS Tissue.

CNS tissue for sens.[a]	Occurrence of CF antibodies[b]	CF antibody titer
Rabbit	11/11	1:64 (mean)
	3/6 (serum pools)	1:32, 1:64, 1:64
Guinea pig	9/13	1:25 (mean)
	2/2 (serum pools)	1:256, 1:64
Hamster	0/14	<1:8
	0/6 (serum pools)	<1:8

[a]One to 4 intracutaneous or footpad injections of spinal cord or brain (hamster) in adjuvant with or without pertussis vaccine.

[b]Sera collected 30 to 43 days after sensitization. Numerator — no. of sera or pools with antibody titer 1:8 or greater; denominator — no. of sera or pools tested.

Production of CF brain antibodies by hamsters sensitized to CNS tissue is shown in Table III. A high proportion of hamsters sensitized to xenogeneic neuroantigen (rabbit or guinea pig CNS) produced CF brain an-

tibodies. Mean antibody titers for groups o individual sera or titers of serum pools ranged from 1:32 to 1:256. Sera from 10 hamsters sensitized to rabbit spinal cord were tested simultaneously against ethanolic extracts of hamster and rabbit brain and found to have identical or very similar titers with either antigen. Despite this demonstration that antigen(s) capable of fixing complement *in vitro* with preformed brain antibodies is present in hamster brain, none of the hamsters sensitized to allogeneic neuroantigen produced significant CF brain antibodies. Furthermore, such antibodies were not demonstrable in sera of 5 hamsters splenectomized prior to sensitization with hamster CNS. CF brain antibody titers in 5 additional splenectomized hamsters sensitized to rabbit CNS were not materially different from those of similarly sensitized nonsplenectomized animals. These observations provide additional evidence that the hamster spleen does not exert suppressive influence on immune responses to neuroantigen.

TABLE IV. Encephalitogenic Activity of Hamster CNS for Hartley Guinea Pigs and Lewis Rats.

Host sensitized	Amount (dry wt) of hamster CNS inj.[a] (mg)	EAE signs	EAE lesions[b]
Guinea pig	3.5	2/15	—
	2.3	2/8	—
	2.0	0/3	3/3
	0.8	0/3	3/3
	0.3	0/3	1/3
	0.1	0/3	1/3
Rat	100	2/3	3/3
	50	5/7	7/7
	25	3/10	9/10
	12.5	0/4	4/4
	6	3/7	6/7
	3	1/3	3/3
	1	0/3	3/3

[a]Reconstituted lyophilized hamster brain emulsified in CFA; 0.1 ml injected into 1 intracutaneous site on anterior neck of guinea pigs and 0.1 ml injected into hindleg footpad of rats, supplemented with pertussis vaccine. For those doses of hamster CNS injected into guinea pigs which exceeded 2 mg, dry weights calculated from amount of 20 or 33% CNS homogenate injected and dry weight of brain being 23% of wet weight.

[b]Guinea pigs sacrificed 17, 32 or 43 days after sensitization; the single group which received 2 injections 45 days apart was sacrificed 22 days after the second injection. Rats sacrificed 16 or 17 days after sensitization except for 4 with paralysis which were sacrificed on the postsensitization day 12 or 13.

Since encephalitogenic neuroantigen in the "CNS target" is essential for development of EAE, we tested hamster CNS tissue to be sure deficiency of encephalitogenic activity was not responsible for the low incidence of EAE in this animal species. Assays of encephalitogenic activity of hamster brain in guinea pigs and Lewis rats are presented in Table IV. In guinea pigs, as little as 2.3 mg (dry wt) induced paralytic clinical signs and 0.1 mg resulted in lesions of EAE. In rats, clinical neurologic signs were observed after sensitization with as little as 3.0 mg. Poor emulsification of inoculum used to sensitize animals at the 12.5 mg dose may well account for the lack of clinical signs. Lesions of EAE were demonstrable in all rats, including those sensitized to the smallest dose assayed, *viz.*, 1.0 mg.

Discussion. EAE can be induced in Syrian hamsters sensitized with either allogeneic or xenogeneic CNS–CFA emulsions (Table I). Clinical signs of the disease, however, are infrequent, mild and transitory. Histopathologic changes of EAE occur in as many as 50 to 60% of sensitized animals in a given experiment. But the lesions tend to be very focal and few in number, often being restricted to a few cellular infiltrates in the spinal cord. In our hands, the hamster has a conspicuous limitation in its capacity to develop EAE when compared with other species of animals used in our laboratories, including rats, guinea pigs, rabbits, dogs and cats [see review Refs. (4, 10)].

Our findings are in direct conflict with those of Tal, Laufer and Behar (2). They reported clinical paralytic signs in close to 25% and CNS inflammatory changes in 100% of Syrian hamsters sensitized to hamster or mouse brain suspensions or hamster proteolipid fractions emulsified in CFA. The majority of their animals were observed for 40 days and some for as long as 140 days. Important differences concerning methodology between their study and ours may account

ences include source of hamsters and status of breeding colonies, type of neuroantigen injected, schedule of immunization, and minor differences in the composition of CFA. It should be noted, however, that a high proportion of the hamsters studied by Tal, Laufer and Behar (2) exhibited clinical signs not ordinarily associated with EAE *per se, viz.*, respiratory difficulty and heavy discharges from the eyes and nose. Even more pertinent, hamsters sensitized to CFA *alone* had inflammatory CNS changes indistinguishable from those observed in animals sensitized to neuroantigen–CFA. Activation of a latent microbial agent under the stress of 6 weekly injections of neuroantigen–CFA or CFA alone would appear to be a distinct possibility. In any case, occurrence of "EAE-like" CNS inflammatory changes in their controls makes it impossible to determine the proportion of hamsters reported by Tal and her associates (2) which developed bona fide EAE

Why does the Syrian hamster have such a low susceptibility to EAE? The experiments using splenectomized and nonsplenectomized animals (Table II) indicate that with respect to immune responses to neuroantigen the hamster spleen does not exert any suppressive influence analogous to that reported for other antigens in rats (12). Immunosuppressive action of splenocytes, therefore, does not appear to account for the low incidence of EAE in Syrian hamsters.

Deficiency in synthesis of antibody, at least as exemplified by CF brain antibodies, also is not an explanation. The majority of our hamsters produced CF brain antibodies following sensitization to xenogeneic CNS tissue (Table III). Other workers (12–16) have reported that "Golden" Syrian hamsters have no conspicuous limitation in antibody production following antigenic stimulation with protein antigens, sheep erythrocytes or microbes. One might raise the question as to whether excessive or accelerated production of CF brain antibodies, which have been shown to have a protective role against development of EAE in rats (17, 18), might have a similar role in hamsters and prevent development of disease. This notion can be excluded, because hamsters which were sensitized to allogeneic CNS tissue did not produce CF brain antibodies (Table III) and they were as insusceptible to EAE as hamsters which did produce antibody after xenogeneic nervous tissue.

Delayed hypersensitivity to neuroantigen is regarded by many investigators as an important immune response in EAE, (10). Cutaneous reactivity to 1:5 and 1:10 dilutions of 20% allogeneic hamster CNS as well as tuberculoprotein (PPD, 125 Tuberculin Units) in 4 hamsters sensitized to guinea pig cord—CFA plus pertussis vaccine was evaluated. All skin test sites were nonreactive at 24 and 48 hr. In previous work with rats (19), intracutaneous injection of CNS suspensions often elicited delayed-type reactions in animals not sensitized to neuroantigens. When such suspensions were diluted so they gave no false positive reactions in normal rats, they usually elicited no significant cutaneous reactions in animals sensitized to neuroantigen. Definitive study of delayed-type cutaneous reactivity to neuroantigen in the hamster will require use of water-soluble defined constituents, *e.g.*, myelin basic protein.

We would stress that we have no reason to believe that the hamster has any defect in the capacity to develop delayed-type hypersensitivity. Hamsters have been used extensively in studies of transplantation immunology and reject skin grafts vigorously, an immunologic event widely accepted as reflecting delayed-type hypersensitivity (3). Kornblum (20) reported that intracutaneous injection of hamsters with lymph node cells from Lewis rats sensitized to neuroantigen together with spinal cord suspensions elicited unequivocal delayed-type inflammatory reactions. These reactions reached maximum intensity at 24–48 hr and were specific for neuroantigen. Hamster skin, thus, would appear to provide a suitable milieu for eliciting delayed-type immunologic reactions. Zakarian, Streilein and Billingham (21) found that isogeneic MHA hamsters sensitized to allogeneic CB hamster tissue gave good delayed-type cutaneous reactions to CB derived poly- for the discrepancy in results. These differ-

oma virus-induced tumor extracts.[4] Biberfeld (16) found that hamsters can produce migration inhibitory factor (MIF), one of the soluble mediators produced by lymphocytes *in vitro* which is closely correlated with delayed-type hypersensitivity.

All of the foregoing studies (3, 16, 20, 21) suggest that hamsters can develop delayed-type hypersensitivity and exhibit the classical *in vivo* and *in vitro* manifestations of this class of immune response. Yet one of the striking observations in our study was the minimal inflammatory response hamsters exhibited to neuroantigen–CFA emulsions and pertussis vaccine. These inocula simply did not elicit the degree of swelling, erythema and other signs of inflammation we expect to observe in other species of animals, *e.g.*, guinea pigs and rats. It may well be that while hamsters can produce MIF, they have a limited capacity to synthesize other soluble mediators essential for generating a vigorous inflammatory reaction to neuroantigen(s). For example, deficiency in production of chemotactic factor or macrophage activation factor might well lead to development of minimal degrees of inflammation at sites of injection of neuroantigen–CFA and also account for the paucity of cellular infiltrates in the "CNS target."

One thing apparent is that difficulty in inducing EAE in the hamster is not due to any gross lack of encephalitogenic neuroantigen in his own CNS. Hamster brain clearly is encephalitogenic for guinea pigs and rats (Table IV). How much encephalitogenic activity hamster CNS possesses, relative to other mammalian nervous tissues, can not be stated without comparative assay data. It is possible that because of neuroanatomic characteristics of hamster nervous tissue, antigenic determinants of encephalitogenic antigen are occluded and unable to interact with sensitized cells and/or antibody. Several workers have raised this question in their *in vitro* studies of localization of myelin basic protein, noting that this neuroantigen does not bind specific antibody well when it resides within intact myelin (22–24).

The cheek pouch of the hamster has been the focus of many studies because it is a privileged site favoring extended survival of tissue allografts and tumors. Billingham and Silvers (3, 25) have shown that the areolar connective tissue layer of the hamster cheek pouch, upon which grafts and tumor implants abut, is unusual in that it impedes graft antigens from reaching draining lymphoid tissues and initiating graft sensitivity. Could the anatomic peculiarity of the cheek pouch also be a structural feature of the hamster CNS? Could the membranes enveloping the hamster CNS impose an unusually restrictive barrier to passage of macromolecules into the neuraxis, well above that of the conventional mammalian "blood–brain barrier"?

Summary. Syrian hamsters sensitized to allogeneic or xenogeneic neuroantigen emulsified in CFA, even with supplemental pertussis vaccine adjuvant, exhibit a low order of susceptibility to EAE. Splenectomy does not alter the pattern of susceptibility. Hamster CNS tissue is encephalitogenic for other species, showing that gross deficiency of "target organ" antigen is not the issue. Hamsters sensitized to xenogeneic CNS produce CF brain antibodies, suggesting that deficiency in production of circulating antibody to neuroantigen is no special problem. Two possibilities are suggested as the basis for the limited capacity of the hamster to develop EAE. One possibility is a deficiency in mediator(s) essential for full-blown inflammatory reactions to neuroantigen. The other possibility is that antigens in intact hamster CNS may be enveloped by unusually restrictive membranes curtailing interaction of immune responses with "target" neuroantigen(s).

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[4] Prompted by this report, describing what appears to be unequivocal delayed-type immune reactivity in MHA hamsters we obtained this inbred strain (from Lakeview Hamster Colony, Newfield, NJ) and tested it for susceptibility to EAE. In a single experiment employing xenogeneic CNS tissue, supplemental pertussis vaccine and the footpad route of sensitization, these MHA hamsters were no more susceptible to EAE than Syrian hamsters.

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