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Regulation of autosensitisation to encephalitogenic myelin basic protein by macrophage-associated and soluble antigen

AUTOIMMUNE diseases are characterised by an attack of an individual's lymphocytes against his own body's components, self-antigens. The immunogenicity of self-antigens can be defined operationally by their capacity to trigger lymphocytes bearing specific receptors. We have designed experiments to learn how a defined self-antigen can function as an immunogen *in vitro* and how the form of its presentation to self-recognising lymphocytes may abrogate this immunogenicity. We have found that myelin basic protein (BP) behaves as a self-immunogen when presented to lymphocytes by way of syngeneic macrophages. Moreover, recognition of this self-immunogen can be inhibited by the presence of the same self-antigen in soluble form during the primary lymphocyte–macrophage interaction.

BP is a self-antigen whose amino acid sequence and encephalitogenic determinants have been characterised[1]. Injection of BP in a suitable adjuvant produces in susceptible animals an autoimmune disease called experimental allergic encephalomyelitis (EAE) (ref. 2). The disease is manifested by histological evidence of lymphocytic infiltration of the central nervous system and is expressed clinically by paralysis. For induction of EAE, BP is usually injected in complete Freund's adjuvant; when the same self-antigen is administered in a soluble form without an adjuvant, it is not encephalitogenic. Moreover, under certain circumstances, treatment with soluble BP or its chemical analogues may suppress EAE (refs 3–5). Thus, the development of EAE seems to require suitable presentation of BP to the immune system.

To analyse the immunogenicity of BP, we focused on the initial phase of lymphocyte recognition of BP and separated this process from the subsequent differentiation of effector lymphocytes. We extended a previous observation that recruitment of immunospecific recipient lymphocytes can serve as a means of detecting whether recognition of self-antigens by initiator lymphocytes has occurred *in vitro*[6]. Thus, the recognition of BP was detected by testing the ability of the initiator lymphocytes to specifically recruit lymphocytes in highly inbred strain 13 guinea pigs. A three-phase system was developed (Fig. 1). In stage I monolayers of macrophages were prepared and pulsed with BP antigen. In stage II, the *in vitro* sensitisation phase, initiator lymphocytes were exposed to the BP-pulsed macrophages in the presence or absence of soluble BP. The initiator lymphocytes were then collected, irradiated to prevent their proliferation, and injected into syngeneic recipient guinea pigs. In stage III, the assay phase, the DNA proliferative response of recruited lymphocytes to BP served to indicate whether autosensitisation of the initiator lymphocytes had occurred.

The results in Fig. 2 demonstrate that BP was recognised, and triggered syngeneic spleen lymphocytes when presented on macrophages. Initiator lymphocytes were exposed to BP-pulsed macrophages and the lymphocytes were injected into recipient animals. Thirteen days later, recruited lymphocytes from the draining popliteal nodes of the recipient animals reacted to BP with a stimulation index (SI) of 4.4 (Fig. 2). The recruited lymphocytes did not respond to lysozyme, a protein of similar molecular weight and charge. Furthermore, lymphocytes incubated on macrophages not pulsed with antigen failed to recruit lymphocytes responsive to either BP or lysozyme, excluding a potential nonspecific stimulation resulting from the lymphocyte–macrophage interaction.

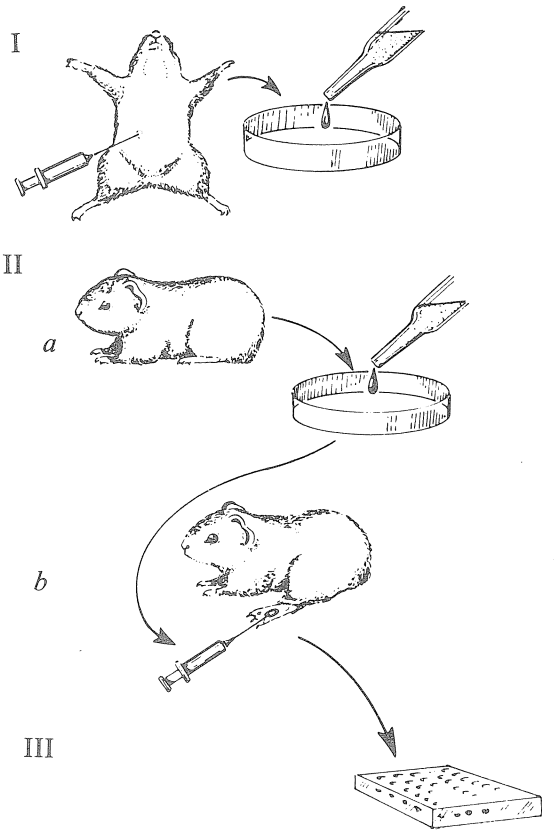

Fig. 1 Experimental format for study of the regulation of autosensitisation to BP. I, BP-pulsed macrophages. Peritoneal exudate macrophages were prepared from unprimed Strain 13 guinea pigs, 4 d after intraperitoneal injection of 15 ml sterile Drakeol, by lavaging the peritoneum with sterile phosphate-buffered saline (PBS) plus heparin, 5 units ml^{-1}. All procedures were then performed in RPMI+25 mM HEPES buffer without serum at 37 °C; 20×10^6 macrophages were allowed to adhere to 100-mm Petri dishes for 2 h, then washed to remove non-adherent cells. The macrophages were then pulsed for one hour with 100 μg ml^{-1} of either BP or G,T. Antigen not associated with macrophages was removed by changing the medium three times. II, Sensitisation of initiator lymphocytes. *a*, 100×10^6 unprimed spleen cells were added to the macrophage monolayers. Some groups received soluble BP at a concentration of 100 μg ml^{-1} during lymphocyte–macrophage interaction. After 16 h of incubation of the lymphocytes on the macrophage monolayers, non-adherent cells were removed, then replated for 1 h to re-adhere detached macrophages. No more than 2.5% of the recovered cells seemed to be macrophages and more than 95% were small lymphocytes. The initiator lymphocytes were irradiated with 1,000 R to prevent their replication but not their ability to recruit *in vivo*[6]. *b*, Lymphocytes were then injected into the footpads of syngeneic animals at a concentration of 10×10^6 cells in 0.1 ml PBS. Some animals received injections of 10^6 antigen-pulsed macrophages in 0.1 ml PBS into the footpads. The macrophages were obtained from the plates by scraping with a rubber policeperson. Each group contained three or four guinea pigs. III, Assay of proliferative response of recruited lymphocytes. After 13 d the draining popliteal nodes were excised and lymph node cell suspensions of each group were pooled. After 1 h of adherence on plastic Petri dishes to remove debris and dead cells, 5×10^5 cells were placed in culture on Greiner microtitre plates. Medium for the assay phase consisted of RPMI+25 mM HEPES+1.25% foetal calf serum. 5×10^4 unprimed, unstimulated macrophages were irradiated with 2,000 R and added to each culture. Cultures were incubated with 15 μg ml^{-1} of various test antigens for 72 h, then 1 μCi of ^{3}H-thymidine was added for 16 h. Cultures were then collected on a MASH 2 cell harvester and counted in scintillation fluid in a Packard Beta counter. Results are reported as the mean and standard error of the four or five culture wells which comprised each group.

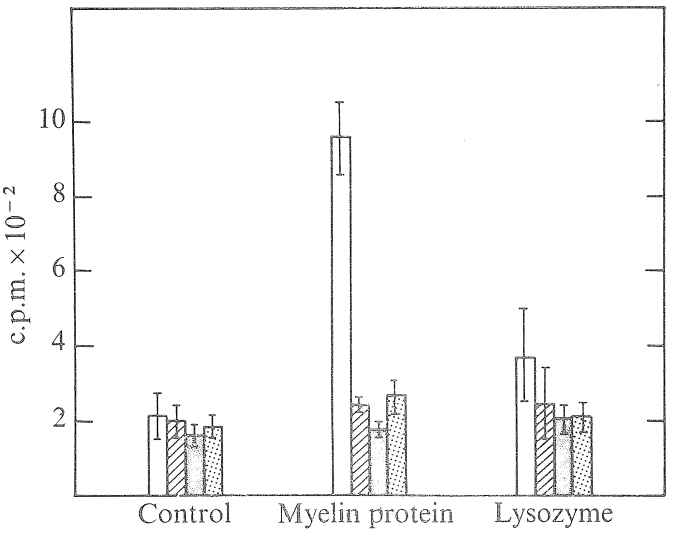

Fig. 2 Immunogenicity of BP on syngeneic macrophages. Incorporation of ^{3}H-thymidine was measured in recruited lymphocytes from the draining popliteal lymph nodes of recipients, 13 d after injection. Recipients received initiator lymphocytes incubated on BP-pulsed macrophages (clear bars) or on macrophages with no antigen (diagonally hatched bars). Other recipients received 10^6 BP-pulsed macrophages (fine stippled bars) or 500 μg of BP directly (coarsely stippled bars). The groups of recruited lymphocytes were incubated without added antigens (control) or with 15 μg ml^{-1} of myelin protein (BP) or lysozyme.

A control was designed to test whether passive transfer of BP, rather than *in vitro* sensitisation of the initiator lymphocytes could account for recruitment of recipient lymphocytes. Guinea pigs were injected with 500 μg of BP, a dose equivalent to the entire amount present in the *in vitro* culture. The recipient animals showed no evidence of sensitisation to BP (Fig. 2), suggesting that mere passive transfer of antigen could not have triggered active sensitisation to BP *in vivo*. Another control was done to test the possibility that antigen-bearing macrophages that had contaminated the injected lymphocytes acted as the initiators of recruitment. Recipients were injected with 1×10^6 macrophages that had been incubated with BP. This number of macrophages was at least 4 times greater than the number of macrophages actually observed to contaminate the injected lymphocytes. The response to this dose of macrophages was also insignificant (Fig. 2). In other experiments (unpublished) we found that injection of 5×10^6 or more BP-pulsed macrophages was needed to sensitise guinea pigs against BP *in vivo*. Thus, autosensitised initiator lymphocytes and not passively transferred antigen nor antigen associated with macrophages induced the recruitment of recipient lymphocytes against BP in these experiments.

Next, we tested whether soluble BP might inhibit the activation of self-recognising initiator lymphocytes by macrophage-bound BP (Fig. 3). Initiator lymphocytes exposed to BP-pulsed macrophages recruited recipient lymphocytes to respond to BP (SI=3.9). However, when initiator lymphocytes were exposed to BP-pulsed macrophages in the presence of 100 μg ml^{-1} of BP in the culture medium, the response of the recruited lymphocytes was markedly reduced (SI=1.6; $P<0.001$). As above, injection of BP-pulsed macrophages (10^6) by themselves failed to recruit a response to BP (SI=1.8).

The inhibitory effect of soluble BP was found to be specific (Fig. 3). Initiator lymphocytes incubated on syngeneic macrophages pulsed with a copolymer of glutamic acid and tyrosine (G,T), recruited a response to G,T (SI=4.7). The presence of 100 μg ml^{-1} of soluble BP did not inhibit this response to G,T (SI=5.4), indicating that the soluble BP was not toxic to lymphocytes. The control injection of G,T-pulsed macrophages did not recruit a response to G,T (SI=12). Thus, the presence of soluble BP specifically blocked the induction of sensitisation to macrophage-bound BP and not to an unrelated antigen.

We have demonstrated that healthy guinea pigs possess lymphocytes that can recognise and be activated by a normal self-constituent, BP, when it is associated with syngeneic macrophages *in vitro*. These lymphocytes, which at least when *in vitro* possess receptors for guinea pig BP, can recruit immunospecific autosensitised lymphocytes in a syngeneic recipient. Indeed, normal as well as immune lymphocytes were observed to bind myelin basic protein, presumably by way of specific receptors[7].

In the experiments reported here we did not observe clinical or pathological signs of EEA in guinea pigs receiving 10^7 initiator lymphocytes. In earlier studies in this laboratory[8], lesions of EAE were produced in rats by the injection of about 4×10^8 initiator lymphocytes of thymic origin sensitised *in vitro* against a crude extract of rat brain. Thus, the development of overt EAE might require injection of many more than the 10^7 initiator lymphocytes sufficient to recruit a detectable proliferative response to BP. Although the conditions for induction of disease were not met, our results indicate that receptor-bearing lymphocytes can be induced to immune differentiation by BP associated with syngeneic macrophages. Furthermore, the presence of the same BP in soluble form can block the activation of lymphocytes by immunogenic BP.

These findings suggest that the mechanism involved in the encephalitogenic activity *in vivo* of BP injected with adjuvant might include activation of host macrophages to present BP lymphocytes in an immunogenic form. It has been shown that adjuvants stimulate macrophages[9]. However, it is not clear how macrophages might render BP

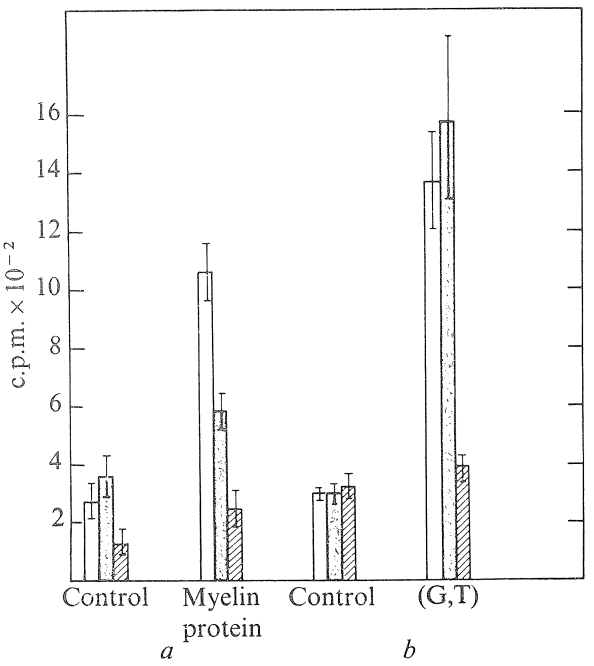

Fig. 3 Blocking of autosensitisation to BP by the presence of soluble BP. Incorporation of ^{3}H-thymidine was measured in recruited lymphocytes from the draining popliteal lymph nodes of recipients 13 d after injection. Recipient animals (Group *a*) received 10×10^6 initiator lymphocytes that had been incubated on BP-pulsed macrophages without (clear bars) or with 100 μg ml^{-1} BP in the culture (stippled bars). Other recipients (diagonally hatched bars) received 10^6 BP-pulsed macrophages. In Group *b* recipients received either 10×10^6 initiator lymphocytes incubated on G,T-pulsed macrophages without (clear bars) or with 100 μg ml^{-1} BP in the culture (stippled bars). Some recipients received 10^6 G,T-pulsed macrophages directly (diagonally hatched bars). The groups of recruited lymphocytes were incubated without added antigens (control) or with 15 μg ml^{-1} of myelin protein (BP) or G,T.

immunogenic for initiator lymphocytes either *in vivo* or *in vitro*. Studies in this laboratory have shown that antigen extracts from syngeneic tumour cells can be immunogenic for mouse lymphocytes *in vitro* when associated with macrophages[10]. Thus, macrophages seem to have an important function in regulating the immunogenicity of "weak" self- or tumour-antigens, as well as that of extraneous foreign antigens[11].

The blocking of immunogenicity by soluble BP is compatible with the concept that non-immunogenic forms of self-antigens might help maintain natural tolerance to self by blocking lymphocyte receptors and preventing their triggering by immunogenic cell-bound forms of the self-antigens[12]. This concept was originally deduced from studies of recognition and triggering of lymphocytes by self-antigens on fibroblasts or thymic reticulum cells[12,13]. The results presented here strengthen this notion as they were obtained without use of heterologous serum which may have influenced the results of the fibroblast or reticulum cell model systems. The use of purified BP as a self-antigen argues against the possibility that sensitisation was directed against unknown viral or embryonic antigens which might have been expressed in cell culture. Furthermore, induction of autosensitisation in the complete absence of heterologous serum rules out any artefacts resulting from possible sensitisation against foreign antigens present in serum[14]. The recent experiments of Wekerle and Begemann, using an *in vitro* model of autosensitisation against testis antigens provides additional support for the concept of blocking self-antigens as regulators of self recognition[15]. It remains to be demonstrated, however, that soluble self-antigens operate physiologically *in vivo* to prevent autoimmunity by blocking recognition of the same self-antigens in immunogenic form.

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1 Hashim, G. A., and Eylar, E. H., *Archs Biochem. Biophys.*, **129**, 645–654 (1969).
2 Paterson, P. Y., *Adv. Immun.*, **5**, 131–208 (1966).
3 Rauch, H. C., Raffel, H. S., and Roboz-Einstein, E., *J. Immun.*, **102**, 1431–1436 (1969).
4 Levine, S., Honig, E. M., and Kies, M. W., *Science*, **161**, 1155–1157 (1968).
5 Teitelbaum, D., Webb, C., Meshorer, A., Arnon, R., and Sela, M., *Nature*, **240**, 564–566 (1972).
6 Cohen, I. R., *Nature new Biol.*, **242**, 60–61 (1973).
7 Yung, L. L. L., Diener, E., McPherson, A., Barton, M. A., and Hyde, H. A., *J. Immun.*, **110**, 1383–1387 (1973).
8 Orgad, S., and Cohen, I. R., *Science*, **183**, 1083–1085 (1974).
9 Unanue, E. R., Askonas, B. A., and Allison, A. C., *J. Immun.*, **103**, 71–77 (1969).
10 Treves, A. J., Schechter, B., Cohen, I. R., and Feldman, M., *J. Immun.*, **116**, 1059–1064 (1976).
11 Unanue, E. R., *Adv. Immun.*, **15**, 95–165 (1972).
12 Cohen, I. R., and Wekerle, H., *J. exp. Med.*, **137**, 224–238 (1973).
13 Wekerle, H., Cohen, I. R., and Feldman, M., *Nature new Biol.*, **241**, 25–26 (1973).
14 Forni, G., and Green, I., *J. Immun.*, **116**, 1561–1565 (1976).
15 Wekerle, H., aud Begemann, M., *J. Immun.*, **116**, 159–177 (1976).

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