CELLULAR IMMUNITY TO ENCEPHALITOGENIC PEPTIDE IN TUMOUR-BEARING MICE

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Summary.—Mice bearing a methylcholanthrene-induced tumour were tested for their cell-mediated reactivity to the experimental allergic encephalomyelitis (EAE) peptide of human myelin basic protein (MBP) in the leucocyte adherence inhibition (LAI) test. Tested over a range of peptide concentrations, peritoneal cells (PC) from tumour-bearing mice exhibited optimal adherence inhibition at 640 ng/ml; PC from normal and parasite-infected mice were unreactive. The EAE peptide also stimulated PC from tumour-bearing mice in the E-rosette augmentation (ERA) test and in the macrophage migration inhibition (MMI) test. MMI appeared to be the most sensitive assay, in that significant reaction at peptide concentrations well below those giving significant LAI and ERA. LAI reactivity to the peptide was detected 5 days after tumour transplantation, and continued to be detectable even with very large tumours. In vitro assays were confirmed by demonstration of EAE peptide recognition in vivo, in tumour-bearing and tumour-excised mice, using the delayed-type hypersensitivity reaction. The present experiments demonstrate an antigenic determinant in murine tumours, similar to the well-characterized EAE peptide of human MBP, and establish an animal model for study and characterization of common tumour-associated antigens.

Since field & caspary (1970) made the surprising discovery that cancer patients’ lymphocytes reacted immunologically with myelin basic protein (MBP), and later showed (Caspary & Field, 1971) that the phenomenon was due to a common tumour antigen, progress in this area has been erratic. Although the basic observation has been confirmed repeatedly with a variety of techniques, practical applications in human cancer are still doubtful and the nature of the proposed common antigen is obscure.

Most of the observations have been made with the macrophage electrophoretic mobility (MEM) technique, either as described by Field & Caspary (1970) or modified in various ways (Pritchard et al., 1973; Dyson & Corbett, 1978). It has been used to demonstrate lymphocyte reactivity in cancer, not only to MBP itself (Caspary & Field, 1971; Rawlins et al., 1976; Chiu et al., 1977) but also to crude and partially purified acid extracts of tumour tissues and cells (Dickinson et al., 1973, 1974, 1980; Carnegie et al., 1973; Shaw et al., 1976). The common antigen of these extracts has been named cancer basic protein (CaBP) because of properties analogous to MBP (Dickinson & Caspary, 1973). Host reactivity to CaBP in human cancer has also been detected by changes in the structuredness of cytoplasmic matrix (SCM) of lymphocytes (Cercek & Cercek, 1977). Both the MEM and SCM assays have nevertheless generated much controversy because of their technical demands (Bagshawe, 1977).

Other techniques have confirmed that lymphocyte sensitization to MBP in cancer is a reality. Thus, extensive studies with macrophage migration inhibition (MMI) revealed that most cancer
patients reacted with MBP (also called encephalitogenic factor, EF) (Flavell & Potter, 1978; Flavell et al., 1978a; Howell et al., 1980) and that lymphocytes of tumour-bearing hamsters and rats were sensitized (Flavell et al., 1978b). Leucocyte adherence inhibition (LAI) also demonstrated reactivity of lymphocytes to MBP in human cancer (Schimke & Ambrosius, 1981).

In contrast to the successful investigations on the structure of MBP and its relationship to experimental allergic encephalomyelitis (EAE) of laboratory animals (reviewed by Hashim, 1978), knowledge concerning CaBP and its role in tumour immunity is incomplete. The molecular region of human MBP containing the encephalitogenic determinant for guinea-pigs has been isolated, characterized and synthesized (Eylar et al., 1970); it is a tryptophan-containing nonapeptide that induces EAE in guinea-pigs but not in rats or monkeys. This EAE peptide was reported to be reactive in MEM with cancer-patients’ lymphocytes (Field et al., 1971; Shaw et al., 1976) suggesting that the cross-reactivity with MBP might depend on a similar peptide determinant in CaBP. Attempts to fractionate and characterize immunologically active fragments of human CaBP have so far been only partially successful (Dickinson et al., 1980).

Advances in the study of CaBP seem to have been retarded by the limitations of the laboratory techniques and the lack of a suitable animal model. The techniques available are tedious and unreliable, and human cancer patients are highly variable. A report on the reactivity of tumour-bearing mice with MBP in the MEM assay (Pasternak et al., 1976) suggested that the cross-reactivity of MBP extended to these animals also, and that they might be suitable for establishing techniques leading finally to applications in man. In our laboratory, the LAI assay has been used extensively with murine and human tumours (Halliday & Miller, 1972; Halliday et al., 1974a, b, 1977; Halliday, 1979; Koppi & Halliday, 1981). It was therefore decided to test the reactivity of the EAE peptide in experimental animal tumours, using LAI and other confirmatory techniques. It was hoped that this would lead to a better understanding of the common antigenic determinants of tumours.

**MATERIALS AND METHODS**

**Animals.**—Inbred CBA mice from the Central Animal Breeding House, University of Queensland, were used when 2–5 months old, and were kept under conventional animal-house conditions.

Nematospiroides dubius infection.—Mice for one experiment were kindly supplied by Dr C. Dobson, Department of Parasitology, University of Queensland. They were infected orally with 100 infective *N. dubius* larvae at 4 weekly intervals (Dobson & Owen, 1977) and were used 4 weeks after the last infection.

**Tumour.**—A fibrosarcoma (MCA-2) previously induced in a CBA mouse with 3-methylcholanthrene was serially transplanted by s.c. inoculation (Maluish & Halliday, 1975). It was in the 16–18th transplant generation at the time of this study. Tumour size was determined by measuring the two greatest opposing diameters after removal from the animal. The transplanted pieces were ~0.5 mm in diameter.

**Tumour extract.**—Extraction with phosphate-buffered saline has been described previously (Halliday et al., 1974, a, b; McCoy et al., 1980); this extract was used in LAI at a final concentration of 1/20, which gave optimal reactivity (Koppi & Halliday, 1981).

**Peptide.**—Synthetic EAE peptide was purchased from Peninsula Laboratories, Inc., San Carlos, Calif., and from Beckman Instruments, Inc., Palo Alto, Calif., USA. It is a segment of human MBP, located at amino-acid residues 114–122, and has the sequence Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg.

**Peritoneal cells (PC).**—The mouse cells used for in vitro assays were obtained from the peritoneal cavity as described by Yong & Halliday (1982).

**In vitro techniques.**—The 3 methods used for assessing cell-mediated immunity (CMI) were direct haemacytometer LAI (Halliday & Miller, 1972; McCoy et al., 1980; Koppi &
Halliday, 1981), indirect ERA (Morrison & Halliday, 1980) and direct MMI (McCoy et al., 1977). In the LAI assay, PC were incubated for 1 h with and without the required antigen, and adherences of cells in these test and control mixtures were determined. The LAI was calculated as the mean % adherence without antigen minus the mean % adherence with antigen. In the ERA assay, PC were incubated for 3.5 h with and without antigen; supernatants were collected and antigen was added at the appropriate level to the control, before storing at −50°C. The test and control supernatants were then tested for their augmenting effect on E-rosette formation by normal human mononuclear cells and SRBC; the ERA value was calculated as the mean % rosettes for the stimulated supernatant minus the mean % rosettes for the control supernatant. In the MMI assay, PC in agarose microdroplets were incubated with and without antigen for 18 h in micro-wells. The migration patterns were then quantitated by projecting through a microscope on to drawing paper, and using a planimeter to estimate the magnified migration areas (Yong & Halliday, 1982). The migration index was the ratio of mean migration area of PC with antigen to the mean migration area without antigen.

Statistical determinations of LAI, ERA and MMI were made by t test comparing mean % adherence, mean % rosettes and mean migration area, respectively, for cells with and without antigen.

Delayed-type hypersensitivity (DTH).—Reactions in mice were elicited by injecting 10 μl of saline containing 40 μg of EAE peptide in one footpad and 10 μl of saline alone in the other (control). Measurements of footpad thickness, before and 24 h after injection, were made with a dial micrometer. Each foot was removed after killing the mice, fixed in 10% formalin, decalcified, and sections were cut, stained with haematoxylin and eosin, and examined for lymphocytic infiltration characteristic of DTH.

RESULTS

LAI reactivity of tumour-bearing mice with EAE peptide

Tumour-bearing mice, known from previous work (Halliday et al., 1974b; Koppi & Halliday, 1981) to have PC specifically reactive with tumour extract, were tested in LAI reactions with a range of concentrations of EAE peptide to establish the dose–response characteristics. The Figure shows the results for 2 experiments with MCA-2 tumour-bearing mice (12 days after transplantation). Over the range of peptide concentrations 80–5000 ng/ml, tumour-bearing PC exhibited increasing LAI up to an optimum at 640 ng/ml, after which inhibition gradually decreased. The maximum LAI values were 14.6 and 17.1 at the optimum, but significant reactivity between 160 and 2500 ng/ml was obtained. The highest and lowest levels of antigen induced no significant LAI.

Normal mice, and mice multiply infected with N. dubius, showed no significant LAI at any peptide concentration (Figure).

Development of reactivity during tumour growth

The MCA-2 tumour was transplanted into 27 mice, and PCs were harvested from groups of 3–5 animals at different stages of tumour growth. These cells were then tested in LAI with optimal concentrations of tumour extract and EAE peptide. As mean tumour diameter
gradually increased, significant reactivity with both antigens appeared 5 days after tumour inoculation, and continued to increase until the experiment was terminated (Table I).

**Comparisons between LAI, ERA and MMI**

PCs from 3 groups of tumour-bearing mice 12 days after transplantation were exposed to a series of concentrations of EAE peptide (as before) and reactivity was assessed by LAI, ERA and MMI assays. The results in Table II show a direct correlation between all tests: the optimal concentrations of antigen were about the same, and reactivity was absent at both low and high concentrations. The MMI assay appeared to be the most sensitive, in that significant reaction occurred at peptide levels well below those giving detectable LAI and ERA. Normal PC had no significant reactivity with EAE peptide in any assay.

**TABLE I.—Kinetics of cell-mediated immune response in mice transplanted with MCA-2 tumour, as detected by LAI against homologous tumour extract and EAE peptide**

| Time after | No. of | Mean tumour diameter extract (mm) | EAE peptide (640 ng/ml) |
|---|---|---|---|
| transplantation (days) | mice in group | (1/20) | |
| 1 | 3 | 0.5 | 3.4 | 3.2 |
| 3 | 3 | 1.8 | 1.0 | 4.8 |
| 5 | 3 | 3.3 | 11.9* | 10.0* |
| 7 | 4 | 6.9 | 14.5** | 12.1* |
| 10 | 5 | 10.3 | 15.8** | 14.8** |
| 12 | 5 | 14.8 | 19.6** | 16.3** |
| 14 | 4 | 15.3 | 19.9** | 20.2** |

† Statistical significance of differences between control and test adherences indicated thus: *P < 0.01; **P < 0.001;

**TABLE II.—Effect of EAE peptide concentration on LAI, ERA and MMI activities of PC from mice bearing MCA-2 tumour**

| Concentration of EAE (ng/ml) | LAI | ERA | MMI index |
|---|---|---|---|
| 5 | ND† | ND | 0.95 |
| 10 | ND | ND | 0.98 |
| 20 | ND | ND | 0.78* |
| 40 | ND | ND | 0.78* |
| 80 | 6.0 | -3.7 | 0.75* |
| 160 | 11.6* | 6.8** | 0.75* |
| 320 | 12.4* | 5.9* | 0.75* |
| 640 | 17.1*** | 5.4* | 0.61*** |
| 1250 | 16.9** | 5.2* | 0.60*** |
| 2500 | 11.0* | 1.8 | 0.64** |
| 5000 | 2.7 | 2.4 | 0.84 |

† not done.

* P < 0.05; ** P < 0.01; *** P < 0.001.

In order to demonstrate recognition of the EAE peptide in vivo, tumour-bearing mice and similar mice after tumour excision were injected in the left hind footpads with peptide solution in saline. Control injections of saline alone were made into the right hind footpad, and an age-matched control group of normal mice was similarly treated. The results of measurements of footpad swelling after 24 h are given in Table III. Animals currently or previously exposed to tumour gave small but significant reactions to the peptide which were not found with normal mice. In another experiment, using a smaller dose of peptide (20 μg), footpad swelling was found at a similar level with tumour-bearing mice, but not with mice after tumour removal (data not shown).

The footpad swellings had the histological characteristics of DTH, as shown by lymphocytic infiltration of the affected tissue of peptide-injected feet compared with saline-injected controls in the same animals.

**DISCUSSION**

In vitro reactions involving a peptide antigen in cancer have previously been confined to the MEM test in man (Field et al., 1971; Shaw et al., 1976) and only the MEM assay had previously been used to demonstrate a cross-reaction between human MBP and murine tumours (Pasternak et al., 1976). The results obtained here with LAI, MMI, ERA and DTH reactions demonstrate the cell-mediated immune (CMI) reactivity of tumour-bearing mice to EAE peptide.
TABLE III.—Footpad reactions (DTH) against EAE peptide in normal mice, in tumour-bearing mice, and in mice after tumour removal

| Mice (n) | Normal (9) | Tumour-bearing* (13) | Tumour-excised† (10) |
|---------|------------|----------------------|----------------------|
| Injection | Saline  | Peptide $\dagger$ | Saline  | Peptide | Saline  | Peptide |
| Mean change in footpad thickness ± s.e. (mm × 10$^{-2}$) | 0·3 ± 0·8 | 0·6 ± 1·0 | -1·1 ± 1·0 | 4·4 ± 1·5 | 0·4 ± 0·4 | 3·5 ± 1·2 |
| $P$        | > 0·5     | < 0·025              | < 0·05               |

* 12 days after tumour transplantation.
† Tumour removed 7 days after transplantation and footpad reactions performed 18 days later.
‡ 40 μg in 10 μl of saline.

Shaw et al. (1976) previously showed that the reaction of lymphocytes from cancer patients with MBP, CaBP and other related proteins was not simply due to their basic nature. They concluded that the lymphocytes were sensitized to determinants shared by MBP and CaBP, and thus responded to these antigens in the MEM test. All the techniques used here involve T lymphocytes, and depend on the presence of receptors on these cells for particular antigenic determinants. The reactions are therefore a consequence of immunological recognition, by tumour-bearing lymphocytes, of an antigenic determinant on the EAE peptide. Furthermore, reactivity of murine PC to the peptide in vitro appeared to be related to sensitization by the tumour, and was not merely the development of unrelated CMI, since PC from normal mice or mice with CMI to N. dubius (Dobson & Owen, 1978) were not reactive. As will be reported in detail in a later paper, positive CMI to the peptide was found in mice of several different strains bearing tumours of different origins.

Determinations of the dose–response characteristics for EAE peptide as antigen in each of the in vitro murine CMI reactions showed that LAI, ERA and MMI possessed different sensitivities. The ability of lymphocytes to react with lower levels of antigen in MMI may be related to the fact that the cells remain in contact with antigen for longer than in the case of LAI or ERA. Inhibition of reactivity by excess antigen was always found and made the use of optimal concentrations essential. This has been reported previous-
producing EAE in guinea-pigs (Hashim, 1978) it might be predicted that CaBP would be common to different tumours in the one species, but exhibit slight changes in amino-acid sequence between species. The actual structure of the immunogenic determinant of murine CaBP must resemble the human EAE peptide, but is not necessarily identical. The conformational requirements for this determinant could possibly be explored by testing a range of synthetic peptides for their activity in vitro with tumour-bearing mouse PC, as has been done in vivo for the EAE-inducing determinant of MBP (Westall et al., 1971; Hashim, 1978; Khanarian et al., 1979).

It should be noted that the EAE peptide is encephalitogenic for guinea-pigs but not for rats, which respond to a different region of MBP (Hashim, 1978). The relevant region in mice has yet to be precisely defined, but may be similar to that in rats (Burgess et al., 1978).

All the in vitro techniques used here have previously been used to investigate lymphocyte reactivity to MBP in human cancer with positive results in most cases (Flavell & Potter, 1978; Schimke & Ambrosius, 1981). A curious exception was found with ERA; a few breast-cancer patients seemed to be unreactive when tested with MBP (Hashim, 1978), though the technique readily detects reactivity to other antigens specific for tumour type (Ramey et al., 1979; Maluish et al., 1980). Further testing of cancer patients using ERA, with materials related to MBP and CaBP, seems to be warranted in view of the simplicity of this technique. Preliminary studies with blood leucocytes from human cancer patients have revealed LAI reactivity to EAE peptide (Maliush & Halliday, unpublished).

CaBP may be only one of a series of common cell markers related to malignant tranformation; another is the p53 protein found in neoplastic cells of mice and other species (Jay & Khoury, 1980). The present identification of an antigenic determinant of a murine tumour, immunogenic in the tumour host and similar to a well-characterized peptide, provides an animal model for the precise definition of the active core of a common tumour-associated antigen.

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