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Measurement by Leukocyte Adherence Inhibition of Autosensitization of Cancer Patients to Myelin Basic Protein

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***In vitro* cell-mediated immunity was assayed by leukocyte adherence inhibition (LAI) to determine the extent of autosensitization to myelin basic protein (MBP). Leukocytes from 123 cancer patients, 16 patients freed of cancer, 135 patients with benign disease, and 26 patients with destruction of nervous parenchyma were tested. Most patients with cancer reacted to MBP: 92%, 93%, 82%, 78%, 75% and 62% for pancreatic, colonic, esophageal, lung, ovarian and breast. Few patients with benign diseases reacted to MBP. Patients with multiple sclerosis (MS) were sensitized to MBP, but patients with other nervous tissue injury did not react to MBP. Cancer patients did not remain sensitized to MBP once they were freed of their cancer. The LAI assay is a straightforward method of measuring cellular autosensitivity to MBP. In the population of patients tested, autosensitivity to MBP was confined, except for MS, principally to cancer patients.**

Key words: Leukocyte adherence inhibition — Myelin basic protein — Human cancer — Cellular immunity — Autosensitization

Experimentally-induced tumors display antigenicity, and a significant fraction of spontaneous animal tumors are potentially antigenic. In fact, many of the early failures to detect antigens on spontaneous tumors[1] reflected the assay methods as much as they reflected the tumors tested.[2–4] From the peripheral blood of patients bearing a variety of common tumors, it has been possible to establish T-cell cultures and T-cell clones capable of lysing autologous tumor cells.[5–7] Antigenicity of a cancer cell may depend not only on the expression of antigenic products but also on the level of MHC molecules available for antigen presentation.[8] In experimental tumors apparently normal cellular products may result in tumor antigenicity[9, 10]; the antigenicity does not need to reflect the expression of mutationally-derived neoantigens or viral antigens.[8]

Three antigenic substances have been identified and defined from human cancers: myelin basic protein (MBP),*[2] T antigen[11] and organ-specific cancer neoantigens (OSN).[12] MBP is major constituent of brain tissue and has 172 amino acid residues (M_r 18,000), and was defined because of its principal role in inducing experimental allergic encephalitis (EAE).[13] Serendipity led Caspary and Field[14] to find that lymphocytes from patients with cancer reacted to MBP. A physicochemically similar protein was acid-extracted from cancer tissue and was named cancer basic protein (CBP).[15–18] A variety of cell-mediated assays confirm that cancer patients are sensitized to MBP.[19–25] In addition, certain groups of patients without cancer are sensitized to MBP.[26] Animals bearing experimental tumors are also sensitized to MBP.[21, 25, 27, 28] The epitopes of MBP responsible for EAE and those that sensitize lymphocytes of animals bearing tumors have been defined.[13, 29]

In the cell-mediated assay of leukocyte adherence inhibition (LAI), MBP is presented in association with class II MHC molecules of monocytes to sensitized T helper lymphocytes ($CD4^+$).[30] This interaction generates leuko-

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*[2] Abbreviations: PBL, peripheral blood leukocytes; LAI, leukocyte adherence inhibition; OSN, organ-specific cancer neoantigen; MBP, myelin basic protein; MS, multiple sclerosis; MHC, major histocompatibility complex; PGE_2, prostaglandin E_2; FCS, fetal calf serum.

triene-like mediators that inhibit the adherence of bystander leukocytes, the assay's endpoint.[30, 31] The present study was undertaken to evaluate the extent of sensitization to MBP in patients with and without cancer. The results showed that most patients with cancer were sensitized to MBP, whereas few patients with inflammatory diseases of the same organs responded to MBP. Patients with multiple sclerosis reacted to MBP, whereas patients with other forms of brain injury did not react to MBP.

Materials and Methods

Patients Patients admitted to hospital because of the suspicion of having cancer were tested. In addition, patients in hospital with benign neoplasms, inflammatory disease or hernias were tested. Most patients were tested shortly after admission to hospital before diagnostic procedures, surgery, or other therapy. In addition, we tested 11 outpatients with well-documented multiple sclerosis and 15 patients on the neurology ward. Sixteen patients with past cancer were referred for follow-up testing of the cancer OSN and were also tested for MBP. Venous blood was collected from each patient in two heparinized 10 ml tubes (Becton, Dickinson and Co. Ltd., Mississauga, Canada).

Leukocytes Buffy coat leukocytes (PBL) were isolated from a 20 ml sample of heparinized venous blood and prepared as previously described.[32] Before being assayed the cells were incubated with $2\times10^{-6}M$ PGE_2 (Sigma Chemical Company, St. Louis, MO) in 1 ml of medium 199 for 5 min at 20° and then diluted to 1×10^7 PBL/ml medium 199.

Computerized Tube LAI Assay The assay, as described by Grosser and Thomson,[32] was performed in 20 ml, 16×150 mm glass test tubes (Kimax) in triplicate. To each tube was added 0.3 ml of medium 199, 0.1 ml of 1% FCS and 0.1 ml of the PBL, and then 10 μl of different concentrations of a stock solution of 1 mg/ml porcine MBP (Calbiochem, La Jolla, CA) in distilled water was added to one row of the tubes (*A*, the specific side). The tubes were agitated, placed in a horizontal position so that the medium covered four-fifths of the lower surface of each tube, and incubated at 37° in a 5% CO_2 humidified atmosphere. After 2 hr, the tubes were placed upright, the medium at the bottom was gently agitated with a Pasteur pipette, and a sample was placed and counted on a specifically marked hemocytometer. The image analyzer (Alpha Omnicon, Bausch and Lomb) counted the cells contained within a 16 mm^2 area of the hemocytometer. The computer then calculated the mean number of nonadherent cells from the three tubes of *A* and of *B* and calculated the nonadherence index:

$$\text{NAI}=\frac{A-B}{B}\times100$$

where *A* equals nonadherent cells in the presence of MBP and *B* equals nonadherent cells in the absence of MBP. Previous studies indicated that less than 5% of control subjects had NAI's ≥30[33, 34]; hence, NAI's ≥30 were considered to be positive.

Results

Dose-response to MBP All tubes contained 1% FCS as a protein source. MBP was added to one of the two sets of three tubes. Since the quantity of MBP added to tubes *A* was less than 1.0 μg, a protein effect was unlikely in the presence of 1% FCS (about 100 μg protein). PBL from subjects without cancer showed no response to concentrations of MBP ranging from 0.1 μg to 0.6 μg/tube (Fig. 1). Higher concentrations were also tested and had no effect (not shown). By contrast, leukocytes from cancer patients gave a sharp positive dose-response curve with a positive response limited to 0.4 μg of MBP,

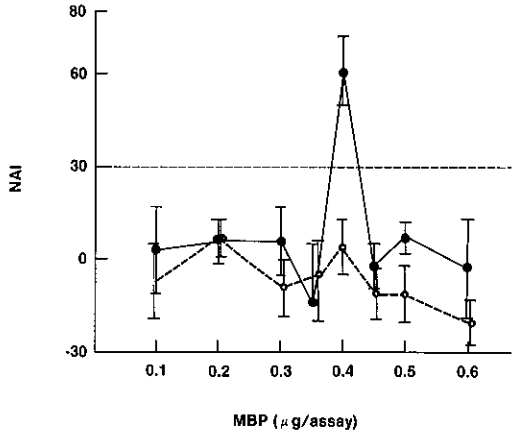

Fig. 1. Dose-response curve of leukocyte nonadherence to MBP. Leukocytes were from patients with cancer other than breast or lung or from patients with benign disease. The nonadherence response (NAI) to MBP was significantly different at 0.4 μg/assay for leukocytes from patients with cancer or benign disease ($P<0.005$). Each point is the mean of a minimum of four assays. Bars indicate SE. Leukocyte donors: ●, cancer patients; ○, control subjects.

similar to that previously reported.[30] The responses of leukocytes from breast or lung cancer patients are not shown in Fig. 1 since they responded best to 0.35 μg and 0.45 μg MBP, respectively.

We previously reported a similar narrow MBP dose-response curve when we studied the mechanism for immune recognition.[30] A peak response was observed with 0.25 μg/assay (0.5 μg/ml).[30] Subsequently, we have observed that the MBP dose-response curve slightly shifts for each researcher, ranging from 0.25 μg to 0.4 μg MBP per assay. Increasing the number of antigen-specific T cell clones in a T cell proliferation assay results in a shift in the antigen dose-response curves towards higher amounts of antigen (i.e. more antigen is required to achieve a given degree of stimulation).[35] In the LAI assay, we believed that changes in cell numbers might slightly shift the MBP dose-response curves. One reason for slight shifts in MBP dose-response curves observed by different researchers may relate to slight but constant differences in the number of leukocytes plated in the assay tubes. When double or triple 1×10^6 cells were plated, positive responses were not observed to 0.4 μg MBP/assay.

Response to MBP of Subjects without Cancer Figure 2 shows the distribution of results with leukocytes from patients without cancer. Of 67 patients with benign conditions such as hernias, cholecystitis or peptic ulcer on the surgical wards, three (4.5%) had a positive test (NAI >30). Of the three positives, one patient had a benign ovarian cyst, another had disseminated granulomatous disease and fever of undefined etiology and the third patient had a hernia. Of twenty patients with diverticulosis and/or diverticulitis, 2 (10%) had positive results. Of 12 patients with pancreatitis without or with pseudocyst, none had a positive response. Of 16 patients with benign breast disease and 4 with benign bladder tumors, none reacted. In summary, few patients with benign or inflammatory disease of parenchymal organs reacted to MBP (Fig. 2).

It was previously reported that certain categories of patients without cancer were sensitized to MBP.[26] Few of these patients were available who were not receiving immunosuppressive treatment with corticosteroids or other drugs. We did test two patients

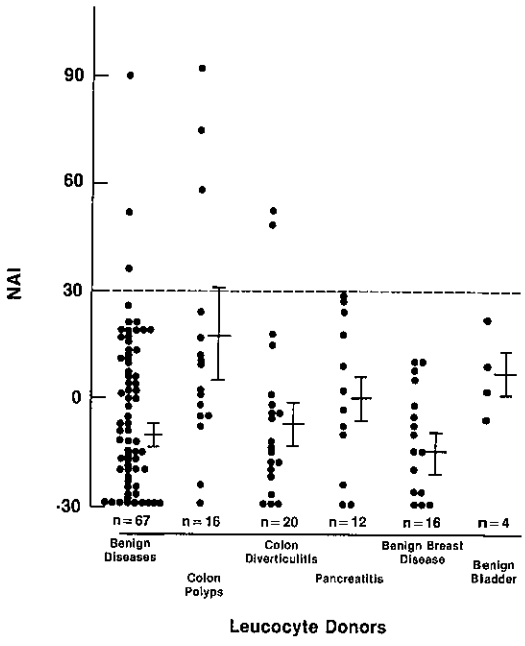

Fig. 2. Distribution of LAI responses to MBP for patients with benign diseases. Values ≥30 are positive.[33,34] n = number of individual patients tested.

with scleroderma, six patients with systemic lupus erythematosus and one patient with Crohn's disease, none of whom reacted to MBP (Fig. 2, Column 1).

Of 16 patients with colon polyps, three (19%) were positive (Fig. 2). The three positive patients had tubular or villo-tubular adenomas. Eight oher patients with adenoma had negative tests. Five patients who had hyperplastic polyps or lesions not classified as adenomas had negative responses.

Response to MBP of Cancer Patients Figure 3 shows the results from testing 109 patients with cancer. Of 13 patients with pancreatic cancer, 12 had positive tests which are in contrast to the negative results of patients with pancreatitis. Of 30 patients with colon cancer, 28 (93%) were positive. Of 11 patients with esophageal cancer, 9 (82%) were positive. Of 18 patients with lung cancer, 14 (78%) had positive responses. Patients with ovarian cancer generally (75%) had positive responses. The lowest percentage of positive responses was observed with breast cancer patients. Of 29 breast cancer patients, 18

(62%) had positive responses (Fig. 3). The explanation for the lower response in breast cancer patients is uncertain; however, we did observe that in general they responded better to slightly lower concentrations of MBP such as 0.35 μg rather than 0.4 μg. Many of the negative results were observed when MBP concentrations of 0.4 μg/assay were used for testing breast cancer patients.

Fourteen other patients with cancer were also tested and are summarized in Table I. All gave positive responses. One ovarian cancer patients had positive tests before chemotherapy and negative tests once chemotherapy was started. Two months after radiotherapy was finished the test was still negative, but four months later the test was again positive. Physical examination showed no recurrence. Of sixteen patients freed of cancer for more than one year, fourteen did not react to MBP, suggesting that MBP sensitization may wane after cancer patients are freed from their cancer. One of the two patients with a positive result presented six months later with a recurrent cancerous nodule in the breast.

Because the leukocytes from patients with early and advanced stages of cancer were treated with PGE_2 before testing, leukocytes from patients with advanced stages of cancer reacted to MBP as well as those from patients with early stages.[32, 34]

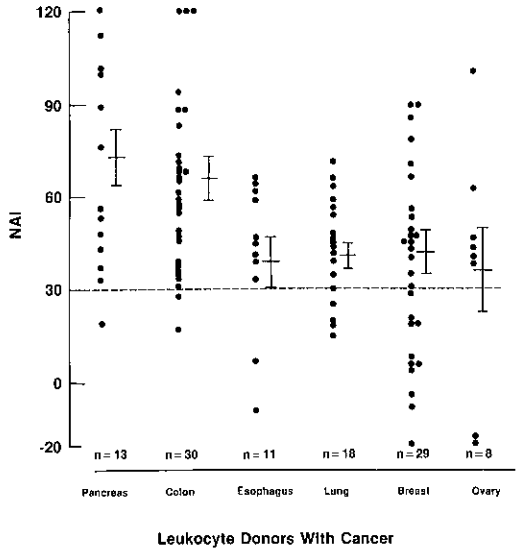

Fig. 3. Distribution of LAI responses to MBP for patients with cancer. Values above the dotted line at 30 are considered positive and those below the line are considered negative.[33, 34] n = number of individual patients tested.

Table I. Results of LAI Assay Testing for MBP Sensitivity for Patients with Other Cancers

Cancer diagnosis of leukocyte donors	Patients tested Number	Positive	Mean NAI
Stomach	5	5	49 ± 8[a]
Prostate	3	3	43 ± 6
Bladder	1	1	38
Liver	1	1	48
Lymphoma in broad ligament	1	1	108
Squamous cell cancer of perineum	1	1	108
Adenocarcinoma of pleural cavity of unknown origin	1	1	46
Testicular lymphoma	1	1	46
Previous cancer now free >1 year	16	2[b]	6 ± 4

a) Mean ± SE.
b) One patient with a positive result, 6 months later, was found to have a cancerous breast nodule.

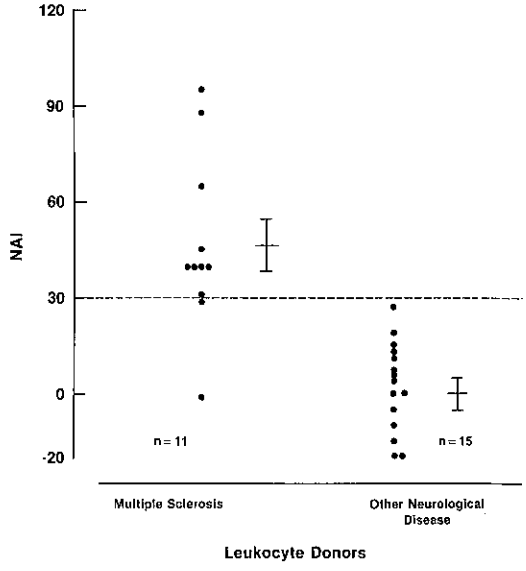

Fig. 4. Distribution of LAI responses to MBP for patients with MS or with other neurological diseases. Values ≥30 are positive.[33, 34] n = number of individual patients tested.

Response of Subjects with Brain Tissue Damage Originally, it was reported that patients with multiple sclerosis (MS) were sensitized to MBP. For this reason, we tested patients with MS as well as patients with other neurological diseases (Fig. 4). Of 11 patients with MS, nine had positive tests. The two negative patients were receiving either corticosteroids or immunosuppressive drugs. All the positive MS patients had received no steroid therapy. Activity or duration of disease had no observable effect on the response.

We expected that other patients with neurological disease might also become sensitized to MBP as a result of brain tissue injury. Fifteen patients with different types of brain or nerve tissue insults, such as strokes, peripheral neuropathy, degenerative neurological disease, intracranial hemorrhage or closed head injury, were tested and were negative (Fig. 4).

Discussion

The results show that most patients with parenchymal cancers reacted in the cell-mediated LAI assay to MBP. By contrast, few hospital subjects with benign disease reacted to MBP. The other group of patients who were clearly sensitized to MBP were patients with MS. Patients with other neurological diseases did not react to MBP. Autoimmunity to MBP seems to be an universal occurrence when cancer develops. The mechanism for the sensitization is unknown.

A series of experiments to be reported explain the reason for the sharp MBP dose-response curves (unpublished results). We found leukocytes to react to a broad range of antigen and in turn to generate the mediator inducing leukocyte nonadherence; however, the generated mediator induces the leukocyte nonadherence behavior only at a narrow concentration range (unpublished results). Likewise, pure chemoattractants induce leukocyte nonadherence at a narrow dose range which is similar to that used for chemotaxis.[31] Nonadherence is used as an index of antigen recognition but the mediator gives an optimum nonadherence response only at limited concentrations; high mediator concentrations actually enhance leukocyte adherence to glass.[31]

The slight shifts in MBP dose-response curves observed in our laboratory[30] by different researchers probably reflect slight but constant differences in the leukocytes plated by each researcher. We have drawn this conclusion for two reasons: first, in T cell proliferation assays, the antigen dose-response curves shift as the number of responding T cells is increased[35]; second, the MBP dose-response curves shifted to negative with increasing cell numbers when the antigen dose was kept constant.

Despite the previous descriptions of MBP-like substances in acid extracts of cancer tissues, MBP is not present in sufficient quantity in crude phosphate-buffered saline extracts as prepared for the LAI assay to trigger a response or to obscure the leukocyte response to the more abundant OSNs. We have estimated that the quantity of MBP is less than 0.1 μg per 100 μg of crude cancer extract (results unpublished). Likewise, Ichinose *et al.*[36] have shown cancer patients to react to pure T antigen in the LAI assay but to give an organ-specific response to crude cancer extracts, even though T antigen is expressed by cancer tissues.[11] Again, the quantity of T antigen compared to OSN in the extracts prepared for the LAI assay is not sufficient to trigger a response.

Field *et al.*[26] reported that patients without cancer who were often sensitized to MBP included those with sarcoidosis, systemic lupus erythematoses, appreciable destruction of nervous parenchyma, myasthenia gravis, Crohn's disease or ulcerative colitis, influenza infections and asthma. Our group of hospitalized control patients included only a few such patients. The reason was that the patients either were unavailable or were receiving therapy. However, of the few patients with Crohn's disease, SLE and scleroderma, that we did test, none reacted to MBP. Of patients with other benign diseases of the colon, stomach, gall bladder or pancreas, few reacted to MBP. By contrast, patients with MS who were not receiving any therapy reacted to MBP. We expected that many patients with nervous tissue damage might become sensitized to MBP. However, of 15 patients with nervous tissue disease or injury who were tested, none reacted. Since Field *et al.*[26] found that sensitization to MBP occurs in many patients with appreciable destruction of nervous tissue, we assume tha the sensitization in patients with other neurological dis-

eases may not be as intense or as prolonged as in MS. Consequently, we may have missed a less intense and shorter sensitization. MS patients' reactivity to MBP did not correlate with disease activity.

Despite immune reactivity to MBP, cancer patients do not develop autoimmune inflammatory brain disease. EAE is induced in laboratory animals by injecting under appropriate immunizing conditions MBP emulsified in Freund's complete adjuvant. The encephalitogenic response is the result of a complex set of interactions that lead to pathologic damage and in mice is under control of genes in the major histocompatibility complex (MHC).[37] Only certain epitopes of MBP induce EAE, and T cells that recognize non-encephalitogenic determinants cannot mediate an autoimmune encephalitis.[37] Further, both encephalitogenic and non-encephalitogenic T cell clones can recognize the same epitope in association with the same class II molecule.[38] Thus, recognition of an encephalitogenic epitope appears to be necessary but is not sufficient for T cell induction of autoimmune encephalomyelitis. An immune attack against human MBP may not occur in healthy brain tissue because MBP does not associate with class I or II MHC molecules on oligodendrocytes to form a target. MBP is an encephalitogenic antigen in post-vaccine encephalomyelitis, as it is in EAE in animals.[39]

The mechanism by which cancer patients become sensitized to MBP is also uncertain. MBP is a highly conserved molecule among different species,[13] and porcine and human MBP have only a few differences in amino acid sequence.[40] The antigenic epitopes of porcine and human MBP are likely to be shared. MBP-reactive T cell lines can be isolated from the peripheral blood of normal individuals[41] and expanded by co-culture with MBP and interleukin 2.[42] Cancer cells may express an altered MBP-like substance which triggers expansion of the MBP-sensitive T cells. Or the cancer process may cause dysregulation of specific suppressor cell activity to induce a local defect in immunoregulation, permitting the functional behavior of existent anti-self effector T lymphocytes.[43,44] Self-reactive lymphocytes are also found in laboratory animals and can be nonspecifically activated *in vitro* during mixed lymphocyte reactions.[45–48] Consequently, it is conceivable that lymphocytes might be activated to initiate an immune response to MBP as part of an unrelated ongoing immune response to other tumor antigens such as the OSNs.

The autoimmune response to MBP as detected by the LAI assay has potential diagnostic value. False positive results were low, ranging from 5 to 10% in patients without cancer. About 19% of patients with colon adenomas had a positive test, but since these lesions are premalignant and should be removed, the result is beneficial. True positive results were high, ranging from 62 to 93%. Consequently, the test detects many patients with cancer. When patients were freed of cancer, autosensitization to MBP waned, indicating the assay's potential use for monitoring the cancer status of patients after treatment. Moreover, detecting autosensitization to MBP has the practical advantage of not having to have available cancer extracts from many different organs.

Many investigators too numerous to completely list have reported good sensitivity and specificity for the LAI assay in diagnosing cancer using cancer extracts[49–62] or T antigen.[36] However, LAI and other cell-based assays used to detect sensitization to either MBP or other tumor antigens require skill and lack built-in standards. Theoretically, the mediators, which are released from antigen-binding leukocytes and are responsible for the changes in bystander leukocyte adherence to glass, should be identifiable and measurable.[31,63] If so, straightforward serological tests for diagnosing cancer might evolve from cellular immune assays but depend on measuring mediators released from antigen-binding leukocytes.

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