Assessment of Immune Responses to Tumors Using Cryostat Sections of Bronchogenic Carcinoma

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ABSTRACT

Thirty patients with lung cancer were studied using the leukocyte migration-inhibition assay. Cryostat sections of autologous and homologous carcinoma tissues and normal lung were used as antigens. Although no inhibition of migration was seen using autologous cancer tissues, seven of 30 patients (23%) demonstrated inhibition of leukocyte migration in response to homologous cancer sections. No association with clinical stage was appreciated. Seven patients demonstrated enhanced migration to homologous malignant tissues; six of these are still alive more than 18 months after testing. The extent of lymphocytic infiltration of tumors was estimated as a possible reflection of host immune response or tumor antigenicity. Patients whose tumors were prominently infiltrated by lymphocytes had a significantly better prognosis than did those whose tumors showed lesser degrees of infiltration. The degree to which a tumor is infiltrated by the host's lymphocytes appears to correlate with survival. Whether or not this correlation is independent of tumor cell type remains to be determined.

INTRODUCTION

The LMI assay has been used by many investigators to demonstrate CMI to antigens from many human and animal tumors including human lung tumors (3, 10, 17, 18, 21). Most of these studies have used soluble extracts from tumor tissue as antigen, and the incidence of positive responses has ranged from 33 to 77% using the authors' own varying criteria of positivity. Most authors did not attempt to relate LMI response and clinical stage although McCoy et al. (10) stated that reactivity in the LMI assay did not seem to be related to either stage of disease or extent of tumor burden at the time of testing.

In 1974, Black et al. (2) reported on the use of the LMI assay to evaluate CMI in patients with breast carcinoma using cryostat sections of breast tissue as antigen. He and his coworkers found a relationship between stage of the disease and LMI response using cryostat sections of autologous breast cancer. They have subsequently used this technique for further antigen purification (22). The cryostat technique offers several advantages: (a) the sections provide a precise microscopic definition of the cellular composition of target tumor tissue and thereby permit more precise correlations between structural and antigenic characteristics; (b) sections are easily prepared and stored; and (c) the technique does not depend on in vitro culture of human tumor cells.

Black et al. (1) and others (8, 9, 16) have also evaluated malignant tumors for in situ reflections of tumor-associated immune reactions. Black et al. reported that lymphocytic infiltration in and around primary breast tumors and the presence of sinus histiocytosis in draining lymph nodes are immune responses associated with enhanced survival.

The present paper reports the application of the modification by Black et al. of the LMI test to assess CMI in 30 patients with lung cancer. Additionally, the tumors of these patients were assessed for the degree of lymphocytic infiltration.

MATERIALS AND METHODS

Patients. Thirty male patients with bronchogenic carcinoma were studied. The mean age was 60 years (range, 46 to 83). Five patients were in clinical Stage I, 3 were in Stage II, 14 were in Stage III, and 8 were tumor free (2 to 36 months after resection). None was receiving immunosuppressive therapy at the time of study; none had received radiation therapy or undergone surgery in the 2 months prior to testing. Twenty patients had squamous cell carcinoma, 6 had adenocarcinoma, and 4 had small-cell carcinoma. No patient was febrile or acutely ill when studied.

Tissue Sections. Cryostat sections, 8 μm thick, were prepared from tumors and normal lung tissues without cryoprotectant and placed on 25-mm coverslip circles for use in Sykes-Moore chambers. Cryostat sections on coverslips were stored at −20° until used. All tissues were obtained at the time of thoracotomy except 4 tumors obtained at postmortem examination within 6 hr of death. Only solid nonnecrotic tumor tissues were cut, and some cryostat sections were regularly stained with hematoxylin and eosin for verification of histological type. No evidence of carcinoma in situ was seen in any of the cryostat sections from normal lung. Sixteen tumors (9 squamous cell, 4 adenocarcinomas, 2 small-cell, and 1 undifferentiated carcinoma) were sectioned as well as 5 normal lung tissues.

LMI Assay. Thirty ml of heparinized blood were sedimented for 30 min with 6 ml of Plasmagel (HTI Corp., Buffalo, N. Y.) at 37°. The leukocyte-rich plasma was then centrifuged at 4° for 25 min at 155 × g, and the cells were washed in CRPMI. All cell culture reagents were obtained from Grand Island Biological Co., Grand Island, N. Y. After recentrifugation, the cells were suspended in 2 ml of 0.9% NaCl solution. Six ml of distilled water were added to lyse the erythrocytes, and 2 ml of 3.5% NaCl solution were added to restore isotonicity. The cells were washed twice and finally resuspended in CRPMI at a concentration of 1.3 × 10⁹ leukocytes/ml. Microcapillary tubes were filled with 20 μl of cell suspension, plugged with Seal-Ease clay (Clay Adams, Canton, Mass.), and centrifuged at 500 × g to produce a 3-mm cell column. The capillary tubes were cut below the cell surface interface and placed into
Inhibition of migration was defined as <84% of control, which represented 2 S.D. below the mean migration of 7 normal persons tested against 36 cryostats of the same malignant sections. Each patient’s leukocytes were tested with an average of 5 different tumors.

**RESULTS**

**Inhibition of Cell Migration.** Nine patients tested against their own tumor sections showed no inhibition of leukocyte migration. Eleven positive responses against homologous carcinoma sections were found in 7 of the 30 patients studied. Clinically, 5 of the responders had Stage III disease, one was Stage I, and one was tumor free. All of the responders had squamous cell carcinoma, and the number of responders with squamous cell carcinoma (7 of 20) differed significantly from the number of responders with non-squamous cell cancer (0 of 10) \( (p < 0.05) \). Responder leukocytes were inhibited by cryostat sections of one adenocarcinoma as well as 4 squamous cell carcinomas. Two patients had positive responses to normal lung tissues. Of the 5 positive responders with Stage III disease, only one had evidence of distant metastases; the other 4 were classified as Stage III by virtue of local extension to the chest wall. Of those 5 patients, 4 have subsequently died. The other 3 positive responders are alive more than 18 months after testing. There was no significant correlation between positive LMI response and clinical stage, presence of distant metastases, or survival.

**Stimulation of Cell Migration.** Cryostat sections from 9 tumors caused stimulation of cell migration \( (MI >1.20) \) in 7 patients. Three patients’ leukocytes were stimulated by their own tumor sections. Of these 9 tumors, 5 induced migration inhibition of the leukocytes of other patients. The additional 4 stimulatory tumors were all squamous cell carcinomas, and, in the group of 16 tumors used as antigens, squamous cell cancer stimulated leukocyte migration more frequently than did non-squamous cell cancers \( (p < 0.005, \) Fisher’s exact test). Except for one patient who was tested preoperatively and died of a cause unrelated to his cancer in the postoperative period, all 7 patients whose leukocytes demonstrated enhanced migration were studied when clinically tumor free 2 to 36 months after resection. They remain alive more than 18 months after testing. Thus, 6 of 7 patients whose leukocyte migration was enhanced, compared with only 8 of 23 patients who never demonstrated enhanced leukocyte migration, are alive at this time \( (p, < 0.025) \).

**LIS.** Among the 17 patients who underwent thoracotomy and from whom adequate tissue was available for determination of a LIS (Table 1), a direct correlation between lymphocytic infiltration and survival was noted. All 9 patients with a LIS of 3 or greater are alive compared with only 3 of 8 patients with a LIS less than 3 \( (p < 0.01, \) Fisher’s exact test).

The LISs of the tumors from patients whose leukocyte migration was inhibited by cancer tissue sections were available in only 3 cases because tissues obtained by fiberoptic bronchoscopy, although sufficient for diagnosis, could not be scored for lymphocytic infiltration. Of the 5 carcinomas, cryostat sections of which induced migration inhibition in others, the LIS was \( \geq 3 \) except for one tumor with an LIS of 1 which was obtained at postmortem examination. The relationship between LIS and ability to induce a positive LMI response among 13 resected tumors used as antigen was examined statistically. The more infiltrated tumors had a significantly greater tendency to induce migration inhibition \( (p < 0.05; \) Fisher’s exact test).

**DISCUSSION**

Of the various in vitro tests used to detect CMI to tumor-associated antigens, none has emerged as being particularly suitable for clinical studies. Even the microcytotoxicity assay \( (6) \) has been discarded because “sequential data in individual patients are difficult to interpret” \( (7) \). The work of Black et al. \( (2) \) with the LMI assay in breast cancer patients is important because of its relevance to the clinical course and prognosis of patients and because of its usefulness in antigen purification and characterization. We therefore thought it important to evaluate the LMI assay using cryostat sections in patients with lung cancer.

In the series of patients with breast cancer studied by Black et al. \( (2) \), 36% of the patients had a MI of \( <0.75 \) when tested against autologous invasive tumor. If a MI of \( <0.85 \) was taken...
as a positive response, 55% of this group demonstrated CMI to these autologous antigens. It was in this group of patients that they demonstrated a clinical correlation; specifically, 90, 46, and 31% of patients with Stage 0, I, and II disease, respectively, had a MI of <0.75. None of the 9 patients tested against autologous tumor sections in this study demonstrated inhibition. Although other investigators (12, 19) using the cryostat technique in breast cancer patients have failed to demonstrate the degree of inhibition described by Black et al., other factors may be responsible for this difference, such as variation in tumor tissues used as antigen.

Using homologous invasive cancer as antigen, Black et al. demonstrated that 15% of breast cancer patients had positive responses when a MI of <0.75 was used and that 34% of patients had a MI of <0.85. They found no correlation between LMI response to homologous cancer sections and clinical stage in their patients. Our frequency of 7 of 30 (23%) is similar to their results. Black et al. specifically point out that, among homologous cancer tissues, cryostat sections of carcinoma in situ seem particularly immunogenic with positive LMI responses seen in 36% (<0.75) and 56% (<0.85) of their patients. Despite attempts to identify only carcinoma in situ in the pathological specimens available from our patients, none was adequate for study.

The positive LMI responses seen in our 7 patients presumably do represent CMI to tumor-associated antigens because positive responses to normal tissues were rare and because normal persons failed to demonstrate any inhibition to cancer sections. The apparent relationship between the ability of a tumor to induce migration inhibition and prominent lymphocytic infiltration corroborates their antigenicity. It appears, however, that other facets of the tumor-induced immune response not reflected in the LMI assay dictate the clinical course of any given patient. It seems reasonable to conclude that the demonstration of inhibition of leukocyte migration to homologous cancer sections will not be helpful clinically in patients with lung cancer in staging or predicting survival. This test may, however, be useful in selecting tumor tissues for further antigen purification and characterization. On the basis of this and other studies, squamous cell cancer associated with prominent lymphocytic infiltration, cryostat sections of which inhibit cell migration in the LMI assay, would be most useful for further study.

The enhanced migration seen in some patients in this series is similar to that reported by other investigators. Seberg (15) as well as others (5, 11, 12, 14) have noted enhancement of migration with various antigens and, although this enhancement has been interpreted by them as evidence of weak antigen sensitization, no specific mechanism has been postulated. In this study, many of the cryostat sections producing enhancement were from the same tumors that in other patients caused migration inhibition, an observation compatible with the hypothesis that this enhancement reflects weak sensitization. Other possible explanations of this observation, however, relate to soluble substances from malignant tumors which have been described to alter the migration of various cell populations (4, 13, 20). Although many of these studies describe the inhibition of cell migration by tumors, Cheung et al. (4) have described a factor from mouse tumors that enhances the migration of peritoneal macrophages. Also, Wei et al. (20) described a nonspecific enhancement of migration of peritoneal exudate cells by cryostat sections of mammary tumors in a mouse model. Although these last 2 factors were demonstrated in systems very different from our own, it is attractive to speculate that similar factors could be operative here. The lack of correlation in this study between the ability of a particular tumor to cause migration enhancement and either LIS (as an in vivo reflection of tumor antigenicity) or survival of the donor suggests that this effect may also be nonspecific. The observed relationship between enhanced leukocyte migration to homologous lung cancer cryostat sections and increased likelihood of survival remains unexplained.

Lastly, the apparent relationship between prominent lymphocytic infiltration of tumor and both the ability of the tumor to induce migration inhibition and also increased survival of the host is interesting. In Underwood’s review (16), many different types of tumors have already been evaluated for lymphocytic infiltration, and a positive prognostic association was found by most investigators. Lung cancer was conspicuously absent from his summary. Ioachim et al. (8) investigated the immune response at the tumor site in lung carcinoma patients. They noted that cellular infiltration was most prominent in well-differentiated squamous cell cancers and least prominent or absent in small cell carcinoma. Although they did not specifically correlate degree of infiltration with clinical course, one could infer a relationship, given the differing biological behaviors of those 2 tumor types. Subsequently, Kaufmann et al. (9) described lymph node changes in patients with operable squamous cell cancers, but they did not examine the primary tumor itself. Although the numbers in our groups are small, the positive correlation between prominent lymphocytic infiltration and survival was significant. If this observation is confirmed, the LIS could become a clinically important means of predicting which resected patients might be candidates for adjuvant chemotherapy or immunotherapy. Whether or not the LIS can provide additional prognostic information independent of cell type is unknown.

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