Environmental Health Perspectives
Vol. 51, pp. 91-96, 1983

Cell-Mediated Cytotoxicity Expressed by Lymphoid Cells from Rats with Asbestos-Induced Peritoneal Mesothelioma towards Rat Fetal Cell

by R. H. Stevens,* D. A. Cole,* H. F. Cheng,* J. A. Hodge* and L. A. Will†

Cell-mediated immunity (CMI) directed towards rat fetal cells was evaluated in Fischer F344 young inbred male rats having asbestos-induced peritoneal mesothelioma. The tumors were induced by exposure to Canadian chrysotile B fibers and the CMI delineated by the injury and destruction brought about to 6- to 10-day-old primary fetal cell cultures by the so-called educated peripheral blood lymphoid-cells (PBLC) obtained from the cancer-bearing rats. A significant cytotoxicity was found to be expressed by the PBLCs, suggesting that during the development of mesothelioma, a cellular retrodifferentiation occurs, thereby educating the effectors to recognize a common determinant existing in both the tumor and fetal cells. Educated PBLCs were produced from rats having endodermal tissue cancers (adenocarcinomas of the small bowel, colon and pancreas) and were found to also be cytotoxic to the fetal cultures, yet no injury was apparently inflicted upon cultured mesothelioma target cells by these effectors. These results suggested that the tumor education was specific and that probably a unique and different fetal component was being recognized by the effector cells obtained from the rats with lesions arising either in the mesodermal or endodermal tissue. Further support for this concept was the failure of an antibody, specific to an oncofetal protein existing in endodermal lesions, to apparently recognize any common oncogenic proteins in the mesothelioma. Preliminary studies have also been accomplished which suggests the existence of natural killing immune responses existing to the mesothelioma target cells. These results, in contrast to other recently reported findings, certainly intimate that asbestos-induced mesothelioma indeed results in host antitumor immune responses and that immune therapy along with immunodiagnosis may in actuality prove to serve an important clinical role in the management of this form of cancer.

Introduction

Numerous biochemical and immunological studies accomplished during the past two decades have extensively documented a close relationship existing on the molecular level between the degree of cellular differentiation and cancer. It was reported in these multifarious studies that cancer cells often possessed many of the molecular properties only associated with the fetal state, thus leading Uriel (*1, 2*) to postulate that cellular differentiation occurring during ontogeny and retrodifferentiation existing in cancer represents an inverse sequence of nucleocytoplasmic events. This resulting expression of fetal characteristics by cancer cells arising in the adult was proposed to represent an alternative of two convergent directions of a single chain of potentially reversible events. Consequently in comparison, the differentiation of a stem cell and retrodifferentiation of a tumor could be assumed traveling through similar stages of nucleocytoplasmic expression, although in an opposite sequence. This process of cancer being a state of retrodifferentiation has been proposed to be a means for morphological and functional simplification which would uniformize originally distinct cell phenotypes and lead to a lower dependency by the tumor on environmental requirements. (*3*).

*Radiation Research Laboratory, Department of Radiology, University of Iowa, Iowa City, IA 52242.
†Department of Veterinarian Microbiology, Iowa State University, Ames, IA 50011.

Furthermore, it has been postulated that the development of fetal characteristics by a tumor is a method whereby the malignant cells, like the fetus, might escape immunological destruction being levied upon it by the host (*4*).

The concept of cancer possessing expressed fetal characteristics while being silent in normal adult differentiated cells has been extensively pursued in an attempt to develop a bioassay for identification of the disease. One of the most widely investigated fetal proteins found existing in cancer has been the carcinoembryonic antigen (CEA), a glycoprotein originally isolated from human colorectal tumors nearly 17 years ago (*5, 6*).

The concentration of this oncofetal protein has been utilized under a variety of circumstances to evaluate clinically both tumor recurrences and the responses to palliative surgery, radiotherapy and chemotherapy of patients having various forms of cancer such as of the colon, pancreas, stomach, biliary tract, breast, cervix, body of the uterus, ovary, lung, and neuroblastoma (*7, 8*). However, recently an analogous study of patients having mesothelioma failed to detect the presence of any carcinoembryoniclike material existing in such tumors (*9*). The investigators concluded that one possible explanation for the lack of CEA being present in such malignant cells was that ontogenetically, mesothelium and endoderm are quite different tissues; consequently, the antibody that was specifically generated to recognize the endodermal (colon) lesions might thus have failed to identify the mesothelioma embryonic-like components. We have recently focused our efforts upon an attempt to establish whether such retrodifferentiation as seen in many other forms of cancer might also occur in the asbestos induced animal model of mesothelioma. Our findings now indicate that rats having such tumors possess immunologically educated peripheral blood lymphoid-cells (PBLCs) capable of recognizing fetal cells, thus suggesting that mesothelioma does undergo a retrogenetic expression and a further search for fetal characteristics as possible tumor markers would be warranted.

Materials and Methods

Tumor Induction

Young adult (200-250 g) male inbred Fischer F344 rats (F344/COBS CD F/CrL BR) were obtained from Charles River Breeding Labs (Wilmington, MA) and provided with commercial laboratory food (Purina Rat Chow) and water *ad libitum*. Tumors were induced by the weekly intraperitoneal injection of 25 mg for 4 weeks (total = 100 mg) of a saline suspension of International Union Against Cancer (UICC) standard reference Canadian chrysotile B, kindly provided by the Medical Research Council, Johannesburg, South Africa. The control animals were similarly injected with a saline suspension of Norite A carbon decolorizing (Fisher Scientific Co., Fairlawn, NJ) and a second group classified as normal rats were exposed to saline only.

Cell Cultures

Primary cell cultures were prepared from 6- to 10-day-old rat fetuses obtained from pregnant Fischer animals. The fetuses were removed under aseptic conditions, washed three times in 100 mL of 50*mM* phosphate-buffered saline (PBS, pH 7.4) containing 100 U penicillin-streptomycin (Grand Island Biological Co., Grand Island, NY), and were finely minced with a sharp scalpel in the culture media consisting of Ham's F-10 containing 10% heat-inactivated fetal calf serum and 100 U penicillin-streptomycin. The tissues were then placed in T-25 Falcon tissue culture flasks (Falcon Division of Becton Dickinson and Co., Oxnard, CA) and the cells were allowed to attach and grow in a humid 95% air-5% CO_2 atmosphere at 37°C. After 5 days, the media and debris were decanted and fresh growth media added to the flask. Confluent layers of cells were regularly obtained within 3 weeks.

Cytotoxicity Assay

The cell-mediated immunity (CMI) was evaluated by quantitating the increased release of radioiodinated peripheral and integral membrane proteins of injured and killed fetal cells brought about by sensitized PBLCs obtained from the test rats as compared with the designated controls. The procedure, which has previously been described in detail (*10*), consists of the following steps: single fetal cells were obtained from the cultures by treatment with 0.25% trypsin in the PBS at 37°C for 5-20 min. A 3.7 MBq (100 μCi) aliquot of carrier-free ^{125}I-Na (Amersham, Arlington Heights, IL), 6.2 nmole of lactoperoxidase (Sigma Chemical Co., St. Louis, MO), and 100 nmole of H_2O_2 in 1 mL PBS were sequentially added to $2\text{-}3 \times 10^6$ fetal cells contained in 1 mL of the PBS and the mixture allowed to incubate for 10 min. The reaction was quenched by the addition of culture media, and the cells were then washed six times with 35 volumes of the media through centrifugation at 400*g* for 15 min in order to remove any unbound radioiodine. Following the final wash, the cells were resuspended to a concentration of 6 to 16×10^3 cells/mL and a 500 μL aliquot placed in each of 24 wells of a Falcon 3008 multiwell test plate. This enzymatic labeling procedure represents a

very mild method for *in vitro* radioiodination, as generally greater that 90% viability (trypan blue staining) and 70% to 80% plating efficiency have been observed for the target cells. Spontaneous release was less than 10% over the 18-hr study interval.

Effector lymphoid cells were obtained from a freely bleeding tail vein of the animals, and the PBLCs were partially purified by sedimentation through a Ficoll-Hypaque gradient (Gallard-Schlesinger, Carle Place, NY) according to the procedures described by Boyum (*11*). The PBLCs were washed with 5 mL of PBS and suspended in the culture media at a lymphoid cell:target cell ratio of 100:1 (6 to 16×10^5 effector cells/mL). A 500 μL aliquot of the effector cells was then added to each well of the plated target cells according to the experimental design for best statistics as described by Brown and co-workers (*12*). Eighteen hours later, three 150 μL samples were removed from each well, and the quantity of iodine-125 released in the form of radiolabeled membrane proteins from the injured and dead target cells was determined by using a Beckman 4000 gamma counter (Beckman Instruments, Irvine, CA). Cytotoxicity was calculated according to the following formulas:

$$\text{Natural CMI} = \frac{(E_c + T) - (T)}{(T)} \times 100$$

$$\text{Tumor CMI} = \frac{(E_T + T) - (E_c + T)}{(E_c + T)} \times 100$$

where the released radioactivity is determined under the designated conditions, with E_T, E_c being, respectively, the effector PBLCs isolated from the mesothelioma and control rats and T represents the radioiodinated target cells. Significance was established by application of a single-tailed Student's t-test.

Results and Discussion

Several studies that have been reported during the past 6 years have noted that mesothelioma cells apparently fail to elicit host antitumor immune responses. For example, Embleton and colleagues (*13*) were unable to detect any cell-mediated immunity using target cultures derived from pleural effusions of patients with pleural mesothelioma and mononuclear effector cells from such individuals. Similarly, Brown et al. (*14*) reported that asbestos-induced transplantable rat mesothelioma cells do not exhibit tumor-associated transplantation antigens. This was deduced by the failure of such cells to bring forth immune responses capable of inhibiting subsequent growth of the transplantable mesothelioma upon rechallenge. It was concluded that this work supported the concept that tumors of long latent periods fail to stimulate host rejection through cell-surface expression of tumor-associated transplantation antigens. In contrast, our recent studies have suggested that such cancer cells do indeed have properties capable of inducing host immune responses, based upon our observations of cell-mediated immunity in rats having intraperitoneal asbestos-induced mesothelioma (*15*). Several factors among the experiments could account for the different observations, such as the method for establishing immunity, whether or not a primary or transplantable tumor model was being investigated, and so forth.

Our current findings now indicate that these so-called tumor-educated PBLCs that are obtained from the asbestos-exposed rats are capable of recognizing in addition to cancer cell components, fetal characteristics as distinguished by significant CMI being expressed towards fetal target cells (Table 1). We contrasted the ability of these effector cells to lyse the fetal cells to two different controls: rats administered charcoal particulate and those injected with isotonic saline. The total CMI expressed is not significantly different when comparing these two types of controls. The charcoal-exposed animals were not seen to possess PBLCs capable of exhibiting any different measurable lytic responses toward such targets when contrasted to the saline exposed rats (Table 2). These observations might possibly be interpreted as the education of PBLCs toward some type of a cellular component which is similar in both the asbestos-induced neoplastic cells as well as existing normally in the fetal cells, i.e., a retrogenetic expression might have occurred in the mesothelioma.

Table 1. Cell-mediated immunity expressed towards rat fetal cells by peripheral blood lymphoid cells obtained from rats bearing asbestos-induced mesothelioma.

	CMI[a]	
Animal	Charcoal	Saline
1	3.3 ± 0.7	6.6 ± 0.6
2	9.2 ± 0.7	14.0 ± 1.1
3	3.8 ± 0.8	2.4 ± 0.9
4	4.9 ± 1.0	1.3 ± 0.6
5	13.3 ± 0.5	— —
Total	6.9 ± 1.9	6.1 ± 2.9

[a]Individual values represent the mean ± SD (N = 36); total is the mean ± SE for the individual animals. CMI calculated by the differences in observed release occurring in the presence of PBLCs from the test compared to charcoal-exposed rats or test compared to normal saline-injected rats. The differences were significant to $p < 0.01$ for all of the individual test rats as compared to their designated controls.

On the basis of our previous studies (*16*) and the present results, we believe that tumor-bearing rats with both gastrointestinal lesions and asbestos-induced mesotheliomas all possess PBLCs generally capable of recognizing fetal cells (Table 3). Apparently, there is a definite specificity that is exhibited by such effectors toward tumor targets, with those effectors obtained from animals having GI tumors (small bowel, colon, pancreas) capable of recognizing only tumor targets derived from endodermal lesions and the PBLCs acquired from rats with mesothelioma recognizing solely the mesodermal-derived targets (Table 4). A large CMI difference was measured for the tumor-bearing rat responses because the animals in the study were not controlled with regards to their tumor burden; however, exact comparisons were made to the corresponding controls with regard to age, size, time since exposure, etc. Consequently, the reported errors appear large for group comparisons, but between individual tumor and its specific control, the CMI was always significant to greater than a $p < 0.01$ level. During the past several years, there have been numerous studies documenting the existence of natural cell-mediated cytotoxicity to tumor cells in mice, rats, and humans (*17*). The subpopulation of lymphocytes found to be responsible for this natural resistance to cancer have been termed NK (natural killer) cells (*18*). Our studies now indicate that such NK effects are also expressed towards mesothelioma target cells (Table 5). While we are currently establishing whether NK cells in addition exert such cytotoxicity towards fetal cells, we can eliminate their contribution to the present results. This is because, in the definition that we have applied for tumor CMI, the NK levels are eliminated through subtraction:

$$\text{Tumor CMI} = \frac{[(\text{specific tumor effects} + \text{NK}) - (\text{NK effects})]}{(\text{NK effects})}$$

Consequently, the values we are now reporting represent increased specific effects brought about by the effector cells obtained from the rats having mesothelioma. Two interpretations can be offered for the fetal cell destruction. One is that the target cells consisted of a mixture containing both endodermal and mesodermal cells, each type having its own unique fetal characteristic common only to that specific type of tumor. A second explanation could be that the effectors were educated to recognize at least two types of cellular components, one found only associated with that specific form of cancer while a second was common to all fetal cells.

In an attempt to differentiate between these two

Table 2. Cell-mediated immunity expressed towards rat fetal cells by peripheral blood lymphoid cells obtained from rats exposed to charcoal.

Animal	CMI[a]
1	0.0
2	0.7 ± 0.7
3	0.0
4	0.0
5	0.7 ± 0.5
Total	0.3 ± 0.2

[a]Cytotoxicity was calculated by the quantity of radioiodinated membrane proteins of the fetal target cells brought about by effector cells obtained from rats exposed to charcoal particulate as compared to animals injected with saline only. Individual values represent the mean ± SE (N = 36); total expresses the mean ± SE for the individual animals.

Table 3. Cell-mediated immunity expressed towards fetal target cells by peripheral blood lymphoid cells obtained from various tumor-bearing rats.

Effector cell source (tumor model)[a]	CMI[b]
Small bowel	7.4 ± 2.1 (N = 4)
Colon	15.1 ± 3.1 (N = 3)
Pancreas	17.9 ± 6.1 (N = 3)
Mesothelioma	6.5 ± 1.6 (N = 9)

[a]Effector cells were obtained from rats having adenocarcinomas induced in the small bowel by X-rays (*22*); in colon by 1,2-dimethylhydrazine (*23*); in pancreas by implantation of 7,12-dimethylbenz[a]anthracene (*24*); and in mesothelioma by the intraperitoneal exposure to asbestos.

[b]Values represent mean ± SE of a group of N animals uncontrolled for tumor burden. Each tumor-bearing rat had significant ($p < 0.01$) CMI compared to its designated control.

Table 4. Cell-mediated immunity expressed towards tumor target cells by peripheral blood lymphoid cells obtained from tumor-bearing rats.

Tumor target cells	Effector cell source (tumor model)[a]			
	Small bowel	Colon	Pancreas	Mesothelioma
Small bowel	20.5 ± 8.3 (7)	–	21.9 ± 19.6 (6)	0.0 ± 0.06 (7)
Colon	8.8 ± 4.0 (7)	–	12.8 ± 6.5 (8)	–
Pancreas	7.4 ± 2.8 (7)	–	7.7 ± 3.1 (6)	0.0 ± 0.07 (5)
Mesothelioma	0.0 ± 0.15 (2)	0.0 ± 0.04 (7)	0.0 ± 0.09 (8)	9.5 ± 2.6 (3)

[a]Adenocarcinomas were induced in the designated tissues as described in Table 3. Values represent the mean ± SE of a group of N rats uncontrolled for their tumor burden.

Table 5. Natural cell-mediated immunity of saline control rats expressed to asbestos-induced mesothelioma target cells.

Animal	CMI[a]
1	26.3 ± 0.5
2	11.2 ± 0.4
3	2.3 ± 0.3
4	8.7 ± 1.7
5	17.9 ± 0.6
6	31.2 ± 0.5
Total	16.2 ± 4.5

[a]Values represent the mean ± SD (N = 36) of individual animals while the total expressed the mean ± for the individual animals.

explanations, we have been utilizing a xenogenic antiserum that specifically recognizes a common tumor-associated fetal antigen existing only in endodermal lesions (*19-21*) in a search for similar fetal proteins in mesothelioma. At the present time, we have been unable to demonstrate that such antibodies are capable of recognizing any such components in the asbestos-induced lesions. Certainly, the failures to date may simply be the result of oncofetal concentration differences and of the titer of the antiserum being such that it is incapable of detecting these fetal proteins in mesothelioma; for this reason a monoclonal antibody to the endodermal oncofetal protein is presently under preparation. It is also very possible that the educated effector cell specifically recognizes a component common solely to the tumor and its fetal cell counterpart; as a consequence, the general fetal cell cytotoxicity may be due simply to a mixture of cell types in the target culture. Wang and co-workers (*9*) similarly had reported they were unable to detect any CEA in patients with mesothelioma when employing an antiserum generated to a colon tumor component. We believe, based upon the results of these studies, that the failures can most likely be attributed to the type of antiserum utilized, and that with the development of the appropriate antibodies generated to the mesothelioma, rather than endodermal cancers, then fetal components will be identified.

Additional evidence has now been provided in this study to implicate a role for antitumor immune responses both specific and nonspecific to asbestos-induced mesothelioma in the rat. There appears to be a retrogenetic expression occurring in the cancer cells as shown by the capability of tumor-educated effector cells to injure normal fetal cells. Whether this represents a general phenomenon for all mesodermal derived tumors remains to be elucidated, and we currently are pursuing similar investigations with other such cancers. Although our work is still inconclusive, we believe that it is quite premature to discount utilizing immunodiagnosis or immunotherapy for patients with mesothelioma.

Authors are indebted to Susan Barnett and Patricia Blaylock for their excellent secretarial assistance in the preparation of this manuscript. This investigation was supported by grants R01-ES02352-03 awarded by the National Institute of Environmental Health Sciences and the March of Dimes Birth Defects Foundation.

REFERENCES

1. Uriel, J. Transitory liver antigens and primary hepatomas in man and rat. Pathol. Biol. 17: 877 (1969).
2. Uriel, J. Foetal characteristics of cancer. In: Cancer: A Comprehensive Treatise, Vol. 3 (F. Becke, Ed.), Plenum Press, New York, 1975, pp. 21-55.
3. Uriel, J. Cancer, retrodifferentiation and the myth of Faust. Cancer Res. 36(11): 4629-4275 (1976).
4. Alexander, P. Escape from immune destruction by the host through shedding of surface antigens: is this a characteristic shared by malignant and embryonic cells? Cancer Res. 34: 2077-2082 (1974).
5. Gold, P., and Freedman, S. O. Demonstration of tumor-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. J. Exptl. Med. 121: 439-462 (1965).
6. Gold, P., and Freedman, S. O. Specific carcino-embryonic antigens of the human digestive system. J. Exptl. Med. 122: 467-481 (1965).
7. Go, V. L. W. Carcinoembryonic antigen: clinical application. Cancer 37: 562 (1976).
8. Bedikian, A. Y. Carcinoembryonic cantigen (CEA) in the clinical diagnosis and treatment of colorectal cancer. Oncology Review, International Cancer Research Data Bank, NCI, Bethesda, MD, 1981.
9. Wang, N., Huang, S., and Gold, P. Absence of carcinoembryonic antigen-like material in mesothelioma. An immunohistochemical differentiation from other lung cancers. Cancer 44: 937-943 (1979).
10. Stevens, R. H., Brooks, G. P., Osborne, J. W., Englund, C. W., and White, D. W. Lymphocyte cytotoxicity in X-irradiation-induced adenocarcinoma of the rat small bowel. 1. Measurement of target cell destruction by release of radioiodinated membrane proteins. J. Natl. Cancer Inst. 59: 1315-1319 (1977).
11. Boyum, A. Separation of blood leukocytes, granulocytes and lymphocytes. Tissue Antigens 4: 269-274 (1974).
12. Brown, J. P., VanBell, G., and Hellstrom, I. Design of experiments using the microcytotoxicity assay. Int. J. Cancer 18: 230-235 (1976).
13. Embleton, M. J., Wagner, J. C., Wagner, M. M. F., Jones, J. P. S., Sheers, G., Oldham, P. D., and Baldwin, R. W. Assessment of cell-mediated immunity to malignant mesothelioma by microcytotoxicity tests. Int. J. Cancer 17: 597-601 (1976).
14. Brown, D. G., Wagner, J. C., and Wagner, M. M. F. Failure to demonstrate tumour-associated transplantation antigens on asbestos-induced mesotheliomas in rats. Brit. J. Cancer 42: 797-801 (1980).
15. Cole, D. A., Stevens, R. H., and Will, L. A. Identification of carcinogens by measurement of cell-mediated immunity. III. Immunity to asbestos-induced rat peritoneal mesotheliomas. Environ. Res. 28: 77-83 (1982).
16. Stevens, R. H., Cole, D. A., and Cheng, H. F. Chemical- and X-ray-induced antitumor cell-mediated immunity to foetal cells. Oncofetal Developmenal Biol. Med. 3: 201-208 (1982).
17. Herberman, R. B., and Holden, H. T. Natural killer cells as antitumor effector cells. J. Natl. Cancer Inst. 62: 441-445 (1979).

18. Herberman, R. B., and Holden, H. T. Natural cell-mediated immunity. Adv. Cancer Res. 27: 305-377 (1978).
19. Stevens, R. H., Englund, C. W., Osborne, J. W., Cheng, H. F., and Richerson, H. F. Oncofetal protein accompanying irradiation-induced small-bowel adenocarcinoma in the rat. J. Natl. Cancer Inst. 55: 1011-1012 (1975).
20. Stevens, R. H., Englund, C. W., Osborne, J. W., Cheng, H. F., and Hoffman, K. L. Identification and characterization of a circulating tumor-associated oncofetal protein from a radiation induced-adenocarcinoma in the rat small bowel. Cancer Res. 36: 3260-3264 (1976).
21. Stevens, R. H., Cole, D. A., and Cheng, H. F. Identification of a common oncofoetal protein in x-ray and chemically induced rat gastrointestinal tumors. Brit. J. Cancer 43: 817 (1981).
22. Coop, K. L., Sharp, J. G., Osborne, J. W., and Zimmerman, G. R. An animal model for the study of small-bowel tumors. Cancer Res. 34: 1487-1494 (1974).
23. Bandara, S., Reddy, S., Narisawa, T., Wright, P., Vukusich, D., Weisburger, S. H., and Wynder, E. L. Colon carcinogenesis with azoxymethane and dimethylhydrazine in germ-free rats. Cancer Res. 35: 287-290 (1975).
24. Stevens, R. H., Graves, J. M., Meek, E. S., Osborne, J. W., Cheng, H. F., and Loven, D. P. Cyclic nucleotide concentrations in 7,12-dimethylbenzo(a)anthracene-induced pancreatic cancer in rats. J. Natl. Cancer Inst. 61: 1281-1284 (1978).