Association between Immunoglobulin and Macrophages in Primary Methylcholanthrene-induced Sarcomas

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ABSTRACT

Ten primary 3-methylcholanthrene-induced murine sarcomas ranging in size from 0.37 to 3.22 ml were selected for studies of tumor-associated immunoglobulin. The purpose of these particular studies was to determine if a significant proportion of the immunoglobulin was bound to receptors for the Fc portion of Immunoglobulin G molecules on tumor-associated host lymphoreticular cells. The distribution of tumor-associated immunoglobulin was compared to the distribution of macrophages within the tumors using indirect immunofluorescence with specific antisera. Significant amounts of AG1, AG2a, AG2b, AG3, lesser amounts of AA, and very little AM were observed. The distribution of each class of immunoglobulin within the tumor was essentially the same, and distribution paralleled the distribution of the tumor-associated macrophages detected with specific antiamphage serum. Double-labeling studies using fluorescein and rhodamine isothiocyanate-conjugated reagents confirmed that the majority of the immunoglobulin was bound to the same cells that were reactive with the antiamphage serum. Furthermore, most of the tumor-associated immunoglobulin, regardless of class, was eluted by washing tissue sections for 5 hr at 37°, a procedure which effectively removes immunoglobulin bound to cellular Fc receptors. B-lymphocytes or plasma cells were rare in the tumors. Those observations provide strong suggestive evidence that most tumor-associated immunoglobulin in primary murine sarcomas is bound to receptors for the Fc portion of Immunoglobulin G molecules on macrophages, possibly in the form of antigen-antibody complexes.

INTRODUCTION

Most studies of the interaction between immunological factors and tumors have involved in vitro analyses of the effects of peripheral factors, e.g., serum antibody, lymph node lymphocytes, or peritoneal macrophages on tumor cells. A relative dearth of information exists with respect to the actual effect of immune factors on progressing neoplasms in vivo.

Only recently has it become clear that there is a significant infiltration of some experimental animal (1, 5, 7, 8, 16, 17) and human tumors (6, 12, 13, 18) by macrophages, K-cells, T-lymphocytes, and granulocytes. Such studies have exposed the possibility that an antitumor immune response is expressed within progressing tumors (4). Also, they have created the necessity for reevaluating certain aspects of previous research in tumor immunology. Specifically, the strong possibility is raised that some TAIg may be associated with IgGFc receptors on infiltrating lymphoreticular cells rather than with antigens on tumor cells. Some of the initial studies have been reviewed recently by Witz (14). There is clear evidence that TAIg is associated with host cells in some tumors (10) while other reports have indicated that both tumor cells and inflammatory cells exhibit bound immunoglobulin (9). Nevertheless, there still is little known about cell association of immunoglobulin in most tumor systems, and even less is known about the functional significance of the TAIg.

Recently, we reported the precise localization of macrophages within primary MCA-induced murine fibrosarcomas (19). That tumor system serves as a model for immunogenic, progressive, rarely metastasizing cancers and offers an excellent opportunity to study the interaction of immunoglobulin with tumor-associated cells. In the present descriptive study, 10 primary murine MCA-induced tumors were selected at random and were stained by indirect immunofluorescence to localize AG1, AG2a, AG2b, AG3, AA, AM, and macrophages. The study was designed to allow semiquantitative determination of the proportion of TAIg that was bound by the Fc as opposed to the amount that was bound by the Fab portion of the immunoglobulin molecule.

MATERIALS AND METHODS

Tumors. Tumors were induced in male C3H mice (Microbiological Associates, Bethesda, Md.) by s.c. injection of 0.5 mg of MCA dissolved in 0.1 ml of triocanoin. Tumors appeared at the s.c. injection site 3 to 5 months following administration of carcinogen and were allowed to progress for 5 to 15 days prior to surgical excision. Tumor tissue was frozen in liquid N2 and stored in sealed vials at −70°.

Antisera. Rabbit antisera to murine immunoglobulins, AG1, AG2a, AG2b, AG3, AA, AM, and M, were purchased from Litton Bionetics, Kensington, Md. Rabbit antimurine macrophage serum was prepared as described previously (19). Briefly, rabbits were immunized with murine macrophages, the resultant serum was absorbed exhaustively with murine kidney and murine thymus homogenates, and its specificity for macrophages was defined by IF on a frozen section of murine thymus, lung, liver, spleen, and on peritoneal exudate cells (17). Furthermore, the antisera exhibited no reactivity for murine immunoglobulin when tested in gel diffusion analyses.

TRITC-conjugated goat antimirine AG was purchased (Cappel Laboratories, Cochranville, Pa.). That antisera was tested in Ouchterlony gel diffusion and found to be cross-reactive.

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with rabbit immunoglobulin. Those cross-reactive antibodies were removed by absorbing the serum with sheep erythrocytes coated with rabbit anti-sheep erythrocyte antibody. Also, the antiserum was unreactive with murine peritoneal exudate cells that had been stripped of their immunoglobulin by trypsination, i.e., it contained no antibodies that were macrophage reactive.

Goat anti-rabbit immunoglobulin was purchased (Miles Laboratories, Kankakee, Ill.) and conjugated with fluorescein isothiocyanate as described previously (15).

The molar fluorescein:protein ratio of the conjugated antiserum was 3.8, and it was always used at a dilution of 1:50.

All reagents were ultracentrifuged (140,000 × g for 60 min) prior to use in order to remove immunoglobulin aggregates.

IIF. Standard IIF methodology was used throughout. Briefly, 4- to 6-μm cryostat-cut sections of tumor were covered with diluted antisera and incubated for 30 min in a moist chamber. The sections were washed for 30 min with PBS, diluted conjugate was added, and the sections were incubated for 30 min longer and finally were washed for 45 min with PBS.

A critical aspect of the IIF methodology involved the prewash step, that is, how the sections were treated prior to the addition of antiserum. Each tumor was tested with all antisera either after no prewash step or after a single 5-hr prewash with PBS at 37°. The purpose of the 37° prewash was to remove IgGFc receptor-associated immunoglobulin (2, 11).

In the routine IIF assays all antisera were used at a 1:50 dilution, i.e., the rabbit anti-murine immunoglobulin was diluted 1:50 and applied, and that was followed by application of the fluorescein conjugated goat anti-rabbit immunoglobulin at 1:50. To ascertain whether maximal sensitivity was obtained at such a dilution, titrations of the antisera were performed. The fluoresceinated reagent had previously been shown to be well within its sensitivity plateau at 1:50; its plateau end point was 1:160. At 1:50, nonspecific background was negligible. Each of the immunoglobulin antisera were titered from 1:10 to 1:160 in doubling dilutions, and the results demonstrated that all of them had similar reactivity profiles, giving maximum fluorescent intensity at 1:40, but were still able to pick up tumor-associated immunoglobulin at 1:160 or higher. There was no change in fluorescent patterns regardless of antisera dilution.

**Double-labeling Immunofluorescence Method.** Concomitant labeling of tumor tissue sections was performed with TRITC-goat antimurine λG and rabbit antimurine macrophage sera to determine the degree of similarity between immunoglobulin and macrophage distribution.

A 3-step protocol was established. TRITC goat anti-murine λG was layered on the tumor section, the section was washed with PBS, rabbit anti-murine macrophage serum (1:50) was added, a wash again was performed with PBS, fluorescein isothiocyanate–goat anti-rabbit immunoglobulin (1:50) was added, and the sections received a final wash with PBS. All steps were 30 min long. Thus, a combined methodology was performed wherein the macrophages were outlined by IIF, and the λG was detected by direct labeling with the rhodamine-conjugated reagent. The key to the success of such a methodology was the high sensitivity of each reagent and the lack of cross-reactivity between any of the reagents.

**Microscopes.** Fluorescence was observed with a Zeiss UV microscope equipped with an HBO200 mercury vapor light source, a Schott BG12 exciter filter, and Schott 65 and 50 barrier filters.

Double labeling with fluorescein and rhodamine was observed with a Zeiss microscope with "Ploem type" optics, a 100-watt quartz iodide light source, and interference filters that were capable of total discrimination between rhodamine and fluorescein. Specifically, the Zeiss Model 4877-10 filter system was used for fluorescein and the Zeiss Model 4877-14 filter system was used for rhodamine.

**Results.** Ten primary MCA-induced tumors varying in size from 0.37 to 3.22 ml were tested for the presence of TAlg by IIF. Significant amounts of λG, λGα, λGββ, and λGβ were present in most or all tumors. Lesser amounts of λA were present in most tumors, and very low levels of λM were detected. There was no evidence of small IgM-positive cells (B-lymphocytes).

Several significant qualitative observations were made with respect to the TAlg. First, all of the TAlg appeared to be cell associated (Fig. 1). Second, not all cells in a particular tumor exhibited fluorescence (Fig. 2). Third, all of the immunoglobulin classes appeared to be bound to the same cells, i.e., the distribution pattern for the immunofluorescent staining was essentially identical for all immunoglobulin classes within a particular tumor. Fourth, often the immunoglobulin staining had a markedly granular character (Fig. 3).

The qualitative nature of the cell-associated immunoglobulin was not highly suggestive of binding to tumor cell-associated membrane antigens. In fact, the staining patterns were similar to macrophage distribution patterns that had been noted in previous studies of primary MCA-induced tumors (19). Further, the fact that each of the immunoglobulin classes exhibited a nearly identical cell distribution pattern suggested the possibility that much of the immunoglobulin was bound to a single cell population. To determine if there was a distributional relationship between Ig's and macrophages in the present 10 tumors, sequential sections from each tumor were stained for macrophages and the various immunoglobulin classes. There were no notable differences between immunoglobulin and macrophage distribution patterns in the 10 tumors (Figs. 4 to 7), suggesting that much of the immunoglobulin was bound to the macrophages.

Double-labeling experiments were performed with each of the primary tumors to determine definitively if the immunoglobulin that was bound within the tumor was bound to the surface of macrophages. Thus, in the same section, macrophages were outlined in green by indirect immunofluorescence with the antimacrophage serum, and λG was detected with TRITC-goat anti-murine λG. It was clear from the onset that it would not be possible to observe distinct points of red and green on individual cells because, as can be seen in Figs. 1 to 12, the staining patterns produced by the anti-λG and antimacrophage reagents were very similar in that both were strong and continuous over the surfaces of the cells. Nevertheless, the following observations that were made on double-labeled sections indicate that the λG and macrophages had virtually identical distribution.

We were able to observe the double-labeled slides on a microscope which exhibited discrimination between rhodamine and fluorescein through the use of properly selected interference filters. To prove to our own satisfaction that such discrimination existed, tumors were labeled separately with either TRITC anti-λG or fluoresceinated antimacrophage serum.
Those sections were then observed with the different filter combinations. When the TRITC-labeled slides were viewed with the filters that selected for the green wavelength, absolutely nothing was seen. It was not even possible to visualize the section. Similarly, when the fluorescein isothiocyanate-labeled slides were viewed for red, nothing was seen. When the double-labeled sections of tumors were viewed for fluorescein and for rhodamine staining, the patterns were virtually identical. In fact, the parallel was even greater than had been anticipated. There were very few cells that stained with one reagent but not with the other. That was true for each of the 10 tumors.

The distributional analyses described above suggested that tumor-associated immunoglobulin was attached to macrophages but provided no information about the binding. If antibodies were present within the tumors and bound to antigens on tumor cells, those bonds generally would be resistant to washing at physiological temperature, pH, and ionic strength. In contrast, if the TAlg was bound by its Fc portion to a cellular IgGFc receptor, that reaction should have been reversible at 37°C (16). Sections from each of the 10 primary tumors were washed at 37°C for 5 hr and then stained for each of the immunoglobulin classes. Except for small areas in three tumors, the TAlg was completely removed by such a manipulation (Figs. 8 to 11). The major exceptions were that some immunoglobulin remained associated with 3 of the tumors and seemed to be in a linear nonmacrophage-associated pattern (Fig. 12), and cytoplasmic immunoglobulin remained in all of the tumors, although it produced relatively weak staining (Fig. 11).

In any type of immunofluorescence study, controls are requisite. In this study, assays with the goat anti-rabbit immunoglobulin conjugate or with the conjugate plus normal rabbit serum uniformly were negative. Those controls established that immunoglobulin molecules in the reagents were not binding nonspecifically to tumor-associated IgGFc receptors. It also was necessary to establish that no cross-reactivity existed between the antimacrophage and the antiimmunoglobulin sera. This was done in 2 ways: (a) the antimacrophage serum failed to react with normal mouse serum in gel diffusion; and (b) peritoneal exudate cells (75% macrophages) were reacted with antiimmunoglobulin and antimacrophage serum in IIF, and both reacted with a proportion of the cells. The cells then were treated with 0.25% trypsin for 30 min and washed. After that treatment, the antiimmunoglobulin reactivity was lost, but the antimacrophage staining was unaffected, thus, clearly establishing that the antimacrophage serum reacted with macrophages but not immunoglobulin, while the antiimmunoglobulin sera reacted with immunoglobulin but not macrophage-associated antigens.

**DISCUSSION**

The existence and significance of antibodies to tumor-associated (specific?) antigens has generated considerable controversy among tumor immunologists. That controversy has been extended to the significance of TAlg. There is no question that TAlg is present in many if not most tumors, and antitumor activity of eluted immunoglobulin has been demonstrated, but the immunoglobulin potentially may be present in the tumor in several forms. First, it may be present as antibody bound to tumor cell-associated antigens. Second, it may be present as uncomplexed immunoglobulin molecules attached to IgGFc receptors on host cells (cytophilic antibody). Those molecules may or may not have antibody activity for tumor-associated antigens. Third, it may be present in the form of antigen–antibody complexes bound to IgGFc receptors on host cells within the tumor. Again, the antigens in the complexes could be tumor associated or totally unrelated. Fourth, it could be present as integral membrane protein on infiltrating B-lymphocytes. Fifth, it could be present as cytoplasmic immunoglobulin of plasmacytes. Sixth, it could be present as endocytosed immunoglobulin. Each of those possibilities was evaluated carefully in the present study.

In the present study of MCA-induced sarcomas, very little of the TAlg appeared to be present as antibody bound to antigens on tumor cells. Areas that were free of mononuclear phagocytes and presumably represented foci of tumor cells were free of immunoglobulin detectable by the method used. In contrast, areas where mononuclear phagocytes were concentrated and detectable immunohistologically (19) contained high concentrations of cell-bound immunoglobulin. Direct proof of association between the immunoglobulin and mononuclear phagocytes was obtained in the present study using specific antigmaphage serum in the double-labeling studies.

Once it was established that the immunoglobulin was macrophage associated, the question of the nature of the binding arose. The 2 major possibilities would be that immunoglobulin was present as complexes bound to cellular Fc receptors on the membrane and as endocytosed immunoglobulin or that it represented antigmaphage antibodies produced coincidently with tumor progression. The fact that over 95% of the TAlg was removable by washing sections under physiological conditions for 5 hr or more recently, by washing sections for 10 min with acetate buffer (pH 5.0) suggested that Fc receptor association was the most reasonable explanation. Previous studies have shown that Fc receptor-associated immunoglobulin is removed readily by washing at 37°C (2, 11). The objection to this experiment is that numerous studies have demonstrated that antibodies bound to antigens on viable cells will dissociate when those cells are cultured at 37°C and that the process is dependent on active cellular metabolism (1, 3, 20). However, cells in frozen sections are metabolically inactive; they have been cut through, frozen, thawed, and air-dried. The antigens on cell membranes in those sections neither cap nor are endocytosed, and antibodies bound to such antigens usually will dissociate completely only at low pH or with high-ionic-strength buffers. An experiment was performed to confirm this with the macrophages in the present tumors. Rabbit anti-mouse macrophage serum was reacted with the sections, the sections were washed for 5 hr at 37°C, for 10 min with acetate buffer (pH 5.0), or for 10 min with glycine buffer (pH 2.5). Only the pH 2.5 elution removed the macrophage-associated immunoglobulin. The other 2 procedures had no apparent effect. In fact, the intensity of staining was greater than in the unwashed control probably because of some reduction in the background produced by the prolonged washing. It is theoretically possible that beyond the macrophage-associated immunoglobulin there was some immunoglobulin bound to tumor cells which were mixed in with the macrophages. The fact of nearly total dissociation under mild conditions also argued against that possibility. In separate studies of human tumors in which there clearly was immunoglobulin bound to tumor cell surfaces, that immun...
noglobulin could only be removed by low pH elution (9). \(^4\)

There was no evidence that B-lymphocytes were present in significant numbers, e.g., no small immunoglobulin-positive cells could be definitively identified in anti-M-stained sections. Also, plasma cells, although present, were very rare.

Most of the TAlg was present in association with macrophages. There was strong evidence in favor of its being present in at least 2 forms, as antigen-antibody complexes on the cell surface and as endocytosed immunoglobulin. The granular nature of the staining suggested the presence of complexes, but the major evidence for immunoglobulin existing in complexed form was 2-fold. First, the quantity of bound immunoglobulin that was detected would be difficult to explain solely on the basis of cytophilic antibody, since the IgGFc receptors on macrophages have low relative affinity for native immunoglobulin. Second, each of the known murine immunoglobulin classes was well represented in association with the macrophages. Since macrophage IgGFc receptors do not have affinity for \(\lambda A\) or \(\lambda M\) and have low affinity for some of the other immunoglobulin classes, there has to be an explanation for their being included in the positive patterns. If one accepts that antibodies in each immunoglobulin class were capable of binding to an antigen, then they would form mixed complexes which would be bound to the macrophages by the Fc portion of some of the \(\lambda G\) molecules, thus accounting for the presence of all immunoglobulin classes in the macrophage-bound immunoglobulin complexes. Of course, one cannot exclude the possibility that some immunoglobulin was bound to the macrophages in a totally uncomplexed form as cytophilic immunoglobulin, and it is possible that low-avidity antimacrophage antibodies were present, although such a phenomenon never has been described.

Those observations raise several questions with respect to the relationship between tumor and TAlg that are worth speculating on. First, where are the antibodies produced? It would appear that the TAlg was produced away from the tumor because B-lymphocytes, and plasma cells were rare in the tumors. Second, if it was not produced within, how did it get in there? Several possibilities suggest themselves. Complexed or uncomplexed immunoglobulin could bind to the macrophages and be carried in as they infiltrated the tumor. Complexes could form in the periphery, infiltrate the tumor, and bind to the tumor-associated macrophages. Alternatively, antibody could infiltrate the tumor, react with soluble tumor-associated antigens, and bind to macrophages, or antibody could infiltrate the tumor and bind to tumor cell-associated antigens. The complexes could then dissociate from the tumor cells and be bound to the macrophages. Third, are the antigens in the complexes derived from the tumor? The answer to that question clearly awaits further experimentation.

REFERENCES


\(^4\) G. W. Wood, unpublished observations.
Fig. 1. Primary Tumor MCA-394 stained by IFF with anti-\(\lambda G_{394}\). \(x 400\).

Fig. 2. Primary Tumor MCA-413 stained by IFF with anti-\(\lambda G_{413}\). \(x 400\).

Fig. 3. Primary Tumor MCA-413 stained by IFF with anti-\(\lambda A\). \(x 400\).

Fig. 4. Primary Tumor MCA-413 stained by IFF with specific anti-murine macrophage serum. \(x 400\).

Fig. 5. Primary Tumor MCA-413 stained by IFF with anti-\(\lambda G_{413}\). \(x 400\).

Fig. 6. Primary Tumor MCA-394 stained by IFF with specific anti-murine macrophage serum. \(x 400\).
Fig. 7. Primary Tumor MCA-394 stained by IFF with anti-λG3. × 400.
Fig. 8. Primary Tumor MCA-394 stained by IFF with anti-λG2b. × 400.
Fig. 9. Primary Tumor MCA-394 stained by IFF with anti-λG2b after tissue section had been washed at 37° for 5 hr. × 400.
Fig. 10. Primary Tumor MCA-394 stained by IFF with anti-λG1. × 400.
Fig. 11. Primary Tumor MCA-393 stained by IFF with anti-λG1 after tissue section had been washed at 37° for 5 hr. Residual cytoplasmic immunoglobulin. × 400.
Fig. 12. Primary Tumor MCA-426 stained by IFF with anti-λA after tissue section had been washed at 37° for 5 hr. × 400.
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