Characterization of Antiserum against Myeloma Cells

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SUMMARY

An antiserum was raised in goats against human malignant plasma cells isolated from a patient with λ-chain myeloma in 1967 and maintained in tissue culture by the Roswell Park Memorial Institute (RPMI 8226). The RPMI cells were harvested, washed, and used without disruption in combination with complete adjuvant for immunization. By electroimmunodiffusion and gel diffusion, the goat anti-myeloma cell serum (AMC) disclosed one and sometimes two immunoprecipitin bands against sonically disrupted RPMI cells. The AMC did not react by electroimmunodiffusion or gel diffusion with human plasma proteins. It also failed to react in gel diffusion and electroimmunodiffusion studies against serum from seven plasma cell cancers (IgG, IgM, IgA, IgE, IgD, and κ and λ chains), serum from three patients with chronic lymphatic leukemia, and four serums showing polyclonal gammapathy. No reaction was found against sonically disrupted preparations from leukocytes removed from normal buffy coat.

The AMC in immunofluorescent studies gave strong membrane reaction with RPMI cells but not with lymphocytes, segmented neutrophils, or intracellular components. Immunofluorescence studies with frozen sections of human liver, spleen, and kidney failed to give a reaction, and no reaction was discernible against three normal bone marrow preparations, seven from patients with myeloma (three IgG κ, two IgG λ, two IgA λ), and four from patients with chronic lymphatic leukemia. There was, however, a strong immunofluorescence at the surface of lymphoid plasma cells in bone marrow preparations from three patients with macroglobulinemia of Waldenström (two IgM κ and one IgM λ). It is unknown at this time whether the AMC depicts a tumor-specific antigen on lymphoid-plasma cells of macroglobulinemia or an antigen in the plasma cell system which is unique to lymphoid-plasma cells. The antiserum does not appear to react with the markers of B lymphocytes.

INTRODUCTION

Immunological evidence is accumulating that some human lymphoid malignant cells contain unique membrane antigens (3, 6–11, 14, 15). Detection of such antigens either on cell surfaces or in body fluids may be of considerable clinical importance. Newer immunological methods are capable of rendering reliable evidence of tumor antigens on cells or in body fluid, thereby permitting elucidation of their earliest appearance during the development of cancer. Studies on the appearance of the tumor antigens will add to an understanding of neoplasia and may open new areas of clinical detection and treatment of cancers. However, before such studies can become effective, the necessary immunological reagents for detection of tumor antigens must be prepared, studied, and evaluated.

This report concerns characterization of AMC2 (2), which appears to detect by immunofluorescence a unique antigen on the surface of the L-P cell in macroglobulinemia and tissue culture myeloma cells. Myeloma cells presumably are products of malignant transformation of PC’s as descendants of B lymphocytes (16) should share surface antigens with B lymphocytes. In the rat both PC’s and B lymphocytes lack the θ antigen, but they do share the PC antigen (18). The AMC, as will be shown, does react by immunofluorescence with the membrane of cultured myeloma cells and with the membrane of L-P cells from 3 patients with macroglobulinemia. No reaction occurred between the AMC and normal lymphocytes or myeloma cells from patients with multiple myeloma, which suggests that at least in this instance the AMC antiserum does not react with a PC-like antigen.

MATERIALS AND METHODS

Myeloma Cells

Myeloma cell culture line (RPMI 8226) was obtained from Roswell Park Memorial Institute (furnished by Dr. George E. Moore). This cell line is now a stable triploid culture. The original cells for this culture were isolated from a patient with λ-chain myeloma (13). The culture line continues to produce λ-chain protein and is not contaminated with Epstein-Barr virus (6). These cells have been maintained in tissue culture at Wesley Medical Center, according to the method described by Matsuoka et al. (13), and were utilized for the production of AMC and as substrate for tests with AMC.

Production of AMC

Both a rabbit and a goat were immunized. The antiserum from the goat is superior, and it is the antiserum used in this study. Washed, intact myeloma cells (200,000 cells/injection)

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2 The abbreviations used are: AMC, goat anti-myeloma cell antiserum; L-P, lymphoid-plasma; PC, plasma cell; RPMI, Roswell Park Memorial Institute; PBS, phosphate-buffered saline; MIF, membrane immunofluorescence.
were mixed with Freund's complete adjuvant and goat polyvalent anti-human serum. The latter antiserum was used in an effort to prevent formation of antibodies to human serum proteins unrelated to the antigens of the membranes of the cultured myeloma cells. The RPMI 8226 cell line produces \(\lambda\)-chain protein (4). The goat does not respond well to \(\alpha\)-chain antigen. Thus, production of goat anti-human \(\lambda\) chain by the injection of cells from a culture line known to produce \(\lambda\) chain was believed unlikely for 2 reasons: (a) use of goat polyvalent anti-human serum; and (b) an animal not readily responsible to \(\lambda\)-chain antigen.

Three i.m. injections of the cells with adjuvant were given at the onset of immunization, at 3 days, and at 14 days. Thereafter, 17 additional i.m. injections were given at 4-day intervals with washed, intact cells (200,000 cells/injection) mixed with equal parts of complete Freund's adjuvant. The last 5 injections contained 500,000 cells/injection. A booster injection of 500,000 cells with complete Freund's adjuvant was given 1 year after start of immunization.

Immunofluorescence Studies

**Direct.** The AMC was conjugated with fluorescein according to the method of Kawamura (5). Separation of the free fluorescein from the conjugate was performed with a P-10 column. The conjugate was further fractionated by DEAE column chromatography. The 1st fraction yielded a conjugate with a ratio of protein-bound fluorescein (\(\mu\)g/ml) to protein (mg/ml) (F/P ratio) of less than 1.

In general, the methods used were those developed in Kelin's laboratory (3, 7–10, 15) for the detection of surface antigens on Burkitt's lymphoma cells. For a routine system of testing living cells, cultured myeloma cells are washed 4 times in PBS and resuspended in the same buffer in a concentration of 10^7 cells/ml. One drop of this cell suspension is placed on a slide and allowed to air dry. The slides that are not used immediately are stored at \(-20^\circ\) for later use. Some cell preparations were fixed in acetone before use. The immunofluorescent reaction is carried out by adding 1 drop of fluorescein-conjugated AMC to the cells on the slide and permitting the slide to incubate at room temperature for 30 min. The fluorescent conjugated serum is removed by washing with 3 changes of PBS, each change lasting 3 min with agitation during washing. The area of the slide with cells is covered with 1 drop of glycerine-PBS mounting fluid and coverslipped.

In suspension of living cells, 1 drop of conjugated AMC is added to 50 \(\mu\)l of living cultured myeloma cells (10^7 cells/ml). After 30 min of incubation at room temperature, cells are washed 3 times with PBS and mounted on a slide as above.

In 1 study, PBS-washed myeloma cells (3 times) were mixed with 2% warm gelatin, and after chilling the embedded cells were sectioned on a cryostat at 6 \(\mu\)m and stained by the direct immunofluorescence method.

Fluorescent examination is performed with a Zeiss microscope equipped with a BG-12 excitor filter and a 53/44 barrier filter.

**Indirect.** The indirect technique is essentially the same as the direct one, except that unlabeled AMC after reacting with substrate preparations is washed from the substrate with 3 changes of PBS. Visual localization is detected with a 2nd antibody made in the rabbit-against-goat IgG, which is labeled with fluorescein. The fluorescent examinations are determined as outlined above.

Characterization of AMC against Serum Proteins and Lymphoid Cells

The AMC was examined by gel diffusion and electroimmunodiffusion (1) against serum proteins from normal and pathological sera. The latter included serum from 7 plasma cell cancers (IgA, IgM, IgG, IgE, IgD, 1 \(\kappa\) chain and 1 \(\lambda\) chain), 3 lymphatic leukemias, and 4 polyclonal gammapathies.

The AMC was evaluated against solubilized membrane fragments from normal cells, buffy-coat cells, and cultured myeloma cells (RPMI 8226) by gel diffusion and electroimmunodiffusion (1). For solubilization, 3 times washed cells were suspended in 1 ml of PBS in a concentration of 10^7 cells/ml and subjected to sonic disruption in a Bronson sonifier set (75 watt) at positive 7 with tuning at 4 amp. The period of sonic disruption lasted for approximately 3 min. Warming was prevented by holding the test tube with the cells in an ice bag.

Application of AMC Antiserum against Human Tissue

Four peripheral blood smears and 2 to 4uffy-coat preparations from each of 6 patients were utilized in this study. Smears of blood and buffy coat were reacted with AMC for direct immunofluorescence studies to determine the reactivity of normal white cells. Three normal bone marrow preparations, 7 from patients with myeloma (3 IgG \(\kappa\), 2 IgG \(\lambda\), and 2 IgA \(\lambda\)), 4 from patients with chronic lymphatic leukemia, and 3 from patients with macroglobulinemia of Waldenström (2 IgM \(\kappa\) and 1 IgM \(\lambda\)) were examined by the direct immunofluorescence method with AMC.

Touch preparations, 1 each from lymph node and frozen sections of spleen, muscle, liver, and kidney, were also examined with AMC by direct immunofluorescence.

Adsorption Studies

Although the AMC was used most frequently without adsorption by human red cells, studies were carried out to delineate the effect of adsorption. Adsorption of the AMC was carried out with human Group O erythrocytes for 18 separate adsorptions until no agglutination of cells occurred, as judged with the aid of a microscope. The antiserum was checked against the cultured myeloma cells to determine reactivity by the direct immunofluorescence method.

An aliquot of adsorbed AMC was further treated with rat liver powder and again reacted with the cultured myeloma cells. Testing with rabbit anti-goat IgG labeled with fluorescein was carried out in addition to the direct method with fluorescein-labeled AMC.
RESULTS

Both direct and indirect immunofluorescence studies with AMC against cultured myeloma cells (RPMI 8226) yielded essentially the same membrane localization of antigen. The same results were obtained with suspension of cells treated with fluorescent reagents and fresh, living cells placed on slides and promptly examined with fluorescent labeled AMC. The acetone fixation of cells was not as successful, and the general method evolved around the use of fresh cells placed on a slide and then reacted with fluorescein-labeled AMC.

Fig. 1 delineates the type of membrane fluorescence observed with cultured myeloma cells. This was clearly delineated by green fluorescence at the membrane by living cells. The membrane reaction was unchanged by repeated absorption of the AMC with Group O erythrocytes. It was abolished by adsorption with cultured myeloma cells. In many instances the pattern of membrane fluorescence was nodular. Some cytoplasmic staining was observed, and polar capping was seen on occasion. Autofluorescence of dead cells was apparent. Against frozen sections of myeloma cells, the localization of the immunological reaction was limited to the membrane with no cytoplasmic reaction.

The AMC did not react by gel diffusion or by electroimmunodiffusion with normal or pathological human sera or tissue culture fluid from the myeloma RPMI 8226 cells which does contain λ chain (13). Fig. 2 shows a double gel diffusion study against solubilized cultured myeloma cells, normal serum, and normal plasma. The AMC reacted only against the cultured myeloma cells with no visible reaction against serum or plasma proteins (Fig. 2) or against pathological sera. No reactions in gel developed against solubilized components of normal lymphocytes or buffy-coat cells.

Electroimmunodiffusion studies with AMC showed no false-positive reactions with PBS and no reaction to normal human serum, but a well-defined reaction with solubilized cultured myeloma cells (Fig. 3).

Direct and indirect immunofluorescence studies with AMC against buffy-coat cells and blood smears from normal patients failed to show a reaction. Three normal bone marrow preparations and bone marrow preparations from 7 cases of multiple myeloma and 4 cases of chronic lymphatic leukemia disclosed no immunofluorescence of any type.

Bone marrow preparations from 3 patients with macroglobulinemia of Waldenström gave the MIF reaction shown in Fig. 4. The fluorescence of the L-P cells is of the rim type which is incomplete.

No evidence of immunofluorescence was detected in touch preparations of normal lymph node and in frozen sections of spleen, muscle, liver, and kidney with AMC.

Exhaustive absorption of the AMC with Group O erythrocytes absorbed the fluorescence. No reaction was seen against frozen sections of myeloma cells. No evidence of immunofluorescence was detected in touch preparations of normal lymph node and in frozen sections of spleen, muscle, liver, and kidney with AMC. Exhaustive absorption of the AMC with Group O erythrocytes absorbed the fluorescence. No reaction was seen against frozen sections of myeloma cells. No evidence of immunofluorescence was detected in touch preparations of normal lymph node and in frozen sections of spleen, muscle, liver, and kidney with AMC.
normal segments of the spleen. This treatment rendered the antiserum specific to Hodgkin's lymphoma cells as shown by reaction against frozen sections of tissue from normal and Hodgkin's lymphoma.

Antiserum to leukemic cell lines (RPMI 6410, myelocytic and A-LKID, lymphocytic leukemia) prepared in rabbits by Tanigaki et al. (20) disclosed multiple antibodies to several antigenic markers on leukemic cell tissues and normal tissue.

In direct reference to myeloma cells, 2 reports are of particular interest. Takahashi et al. (18, 19) demonstrated with mouse antiserum to mouse myeloma cells that immunoglobulin-producing cells or bursa-derived cells (B-cells) are clearly different from the thymocytic-lymphocytic cells (T-cells). The B- and T-cells have distinct alloantigens but share alloantigens of the H-2 type. An antigen unique to the PC designated PC. 1 is found on normal and malignant PC's and is shared with the kidney, brain, and lymph nodes. Antiserum to myeloma cells reacts with the hemolytic plaque-forming cells of the spleen.

Watanabe et al. (21), using an antiserum made in rabbits against sediments of BALB/c mouse myeloma cells, showed that it reacted with the surface of plaque-forming cells (normal PC's) and malignant PC's. This antiserum was cytotoxic to myeloma cells but not to lymph node cells or splenic cells. Suppression of the plaque-forming cells was removed by absorption of the antiserum with myeloma cells. In contrast, antiserum to lymph nodes, when absorbed with myeloma cells, did not suppress the activity of the antiserum against lymphocytes.

**DISCUSSION**

The most extensive work on human lymphoid cells associated with tumor antigens is that reported from Klein's laboratory on Burkitt's lymphoma (3, 6–11, 14, 15). Klein and associates, using direct and indirect immunofluorescence antibody techniques with serum from patients with antibodies to Burkitt's lymphoma cells, were able to show that the living lymphoma cells from tissue culture or from biopsy carry on their surface an antigen unique to the Burkitt's lymphoma cell. The ring-like pattern of MIF was established as a property of the membrane antigen.

In some serum, antibodies to Epstein-Barr viruses are present. These also react with the membrane of Burkitt's lymphoma cells. In infectious mononucleosis, patient's serum will also react with the Burkitt's lymphoma cells to form MIF. However, by cross-absorption techniques, Klein et al. (8) have shown that the MIF in Burkitt's lymphoma disclosed by antibodies derived from patients with Burkitt's lymphoma are specific to a membrane antigen that is distinct from that of the Epstein-Barr virus or of infectious mononucleosis.

A recent report by Order et al. (14) contains evidence of a tumor-associated cellular antigen in Hodgkin's disease. These authors prepared antiserum in rabbits against Hodgkin's cells harvested from spleens removed from patients with active Hodgkin's disease. The antiserum was cross-absorbed with
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Fig. 4. Direct immunofluorescence study on a patient with macroglobulinemia of Waldenström. The spotty rim localization of the AMC is well demonstrated in the cells shown and is consistent with a pattern of rim fluorescence. X 400.

nodes. The antiserum to lymph nodes did not suppress plaque-forming cell reaction.

These 2 studies would indicate that PC's and myeloma cells in the animals studied react with the serum prepared against myeloma cells and thus presumably share common surface antigens. These studies tend to suggest that the PC. 1 antigen in the mouse is not unique to tumor cells (myeloma cells) and thus would not be classified as a tumor-specific antigen.

To our knowledge, no report concerning tumor-specific antigens on myeloma cells of human origin or anti-myeloma antibodies or tumor-specific antigens in serum of patients with myeloma or macroglobulinemia of Waldenström has appeared in the literature. The results of our study with AMC indicate that the antiserum does not react with normal or pathological serum proteins but does react with the tissue culture myeloma cells to form MIF, a characteristic of membrane localization of antigen(s). The anodic electrophoretic mobility of the myeloma cell antigen as depicted by electroimmunodiffusion lends support to the membrane as the origin of the myeloma antigen. Although cytoplasmic proteins may also have a relatively high negative charge at pH 8.6, the membrane proteins are much more likely to be more negatively charged (1). Of note are the results of the frozen section studies on the myeloma cells, which showed no cytoplasmic localization, only membrane localization. The antiserum fails to react with cells from normal buffy coat, myeloma cells, or cells from lymphatic leukemia. The \( \lambda \) chain produced by the RPMI 8226 cell line is nonreactive with the AMC. This finding is indicative of successful blocking of anti-\( \lambda \) production in the goat by use of goat anti-human antiserum mixed with the myeloma cells at the time of immunization. The antiserum does, however, react with human erythrocytes. This is also true of normal goat serum. Absorption of the AMC with red cells does not remove the antibody to the membrane of cultured myeloma cells.

Three cases of macroglobulinemia of Waldenström clearly show a distinctive pattern of MIF of the L-P cells. Marmont and Damasio (12) showed that B lymphocytes are demonstrated in macroglobulinemia by immunofluorescence with anti-\( \mu \) antiserum. The anti-AMC does not appear to be directed at the \( \mu \) marker of B lymphocytes, since no reaction was detected against lymphocytes from patients with chronic lymphocytic leukemia. It remains to be seen whether the AMC depicts a tumor-specific antigen on the L-P cells of macroglobulinemia or an antigen in the PC system that is unique to L-P cells. The antiserum, hopefully, could be used to delineate cell lines in tissue biopsies to aid in establishing a diagnosis more rapidly than is now possible in certain types of PC cancers (17).
REFERENCES

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