Independence of Chicken Major Histocompatibility Antigens and Tumor-Associated Antigen on the Surface of Herpesvirus-Induced Lymphoma Cells

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Capping of chicken major histocompatibility (MHC) antigens on normal thymus, spleen, and peripheral blood leukocytes was demonstrated, although MHC antigens appeared to be present on only 15 to 18% of normal thymus cells. MHC antigen capping also occurred on cells from a Marek’s disease herpesvirus-induced transplantable lymphoma (MDCT-NYM1). Capping of a Marek’s disease tumor-associated surface antigen (MATSA) could be induced on MDCT-NYM1 lymphoma cells as well as on cells of two Marek’s disease in vitro lymphoblastoid cell lines (MDCC-MSB1 and MDCC-LS1). Cocapping of MHC antigens and MATSA did not occur on MDCT-NYM1 lymphoma cells. The results suggest that MHC antigens and MATSA are not structurally associated on the cell membrane.

Major histocompatibility (MHC) antigens have an important role in the cell-mediated immunological defense against aberrant syngeneic cells. Studies with mice suggest that the reaction of cytotoxic thymus-derived lymphocytes (T cells) against virus-infected target cells involves recognition of both viral antigens and histocompatibility antigens (35). The requirement for H-2 compatibility between cytotoxic T cells and target cells indicates a histocompatibility restriction with regard to recognition by T cell receptors. A possible basis for histocompatibility restriction of the mouse cytotoxic T cell response against Friend virus-infected cells has been demonstrated (3). Resistance to virus-infected cells is determined by the mouse H-2 type and is correlated with the apparent formation of an H-2-viral antigen molecular “complex” on the cell surface. This study showed that the complex, which elicits a strong cytotoxic T cell response, is associated with the H-2D\(^b\) haplotype.

Whether or not products of the MHC are involved in T cell-mediated immunity against tumor cell antigens is unclear since conflicting results have been reported. With some mouse tumors, H-2 antigens may play an important recognition role in tumor cell killing by syngeneic T cells (8, 35). In contrast, histocompatibility restriction was not observed in response to oncostetal (7), plasmacytoma (7), or teratocarcinoma antigens (2).

Evidence has been obtained for histocompatibility restriction in the immune response by chickens to Rous sarcomas (24, 32). Other studies have shown that regression of Rous sarcomas is under MHC gene control (9, 23). In addition, resistance of chickens to Marek’s disease herpesvirus (MDV)-induced lymphomas is, at least in part, under MHC gene control since the B\(^21\) haplotype appears to confer resistance to Marek’s disease (MD) (4, 16, 18). Therefore, histocompatibility restrictions may also regulate the T cell-mediated response to MDV.

Numerous studies have been performed in attempts to verify either the one or the two T cell receptor models proposed as a basis for the histocompatibility restriction phenomenon (35). Such investigations have focused on whether the association between viral or tumor-associated antigens and histocompatibility antigens results in a neoantigen or antigen complex on the cell surface which could be recognized by a single T cell receptor (5, 11, 12, 14, 34a, 35).

One method used to study the cell surface association between antigens, and the possible formation of antigen complexes, involves capping and cocapping, i.e., antibody-induced redistribution of antigens into a localized area on the cell surface which is recognized by fluorescent microscopy (30). Capping has been reported for histocompatibility antigens on the surface of normal mouse (20, 28) and human lymphoid cells (17). Cocapping of viral antigens with histocompatibility antigens was found for EL4 and Rauscher leukemia cells (25, 34a) and cell lines derived from spleens of mice infected with Friend leukemia virus (5). Cocapping of tumor-associated antigens and histocompatibility antigens was observed with cells from testicular teratomas in mice (14) and a virus-transformed osteosarcoma in rats (11).

In the present study, we examined capping of
chicken MHC antigens on normal cells as well as MDV-induced lymphoma cells. An investigation was also made to determine if there was cocapping of MHC antigens with a Marek's disease tumor-associated surface antigen, previously designated MATSA (34).

MATERIALS AND METHODS

Animals. Chickens used in this study were from two closely related inbred White Leghorn lines (G-B1 and G-B2) which differ at the B blood group histocompatibility complex (the chicken MHC). Lines G-B1 and G-B2 possess MHC haplotypes designated B' and B², respectively. Haplotypes designated B' and B² in other lines of chickens may represent different MHC antigens. All experimental birds were kept in isolation units of the Horsfall-Bauer type with filtered air pressure.

Cells. Normal thymus and spleen cells as well as peripheral blood leukocytes (PBL) were taken from uninfected G-B1 and G-B2 birds. The PBL were obtained from heparinized blood by separation over Ficoll-Hypaque. Cells from an MDV-induced transplantable lymphoma were used. This lymphoma was developed in line G-B1 by using the Conn B isolate of MDV (31) and is designated MDCT-NYM1 according to the recently proposed nomenclature system (33). The lymphoma was propagated by serial passage in the pectoral muscle of 2- to 4-week-old G-B1 birds.

Tumors, thymus, and spleens were aseptically removed and made into single cell suspensions in RPMI 1640 medium as previously described (31). Mononuclear lymphocytes were obtained from spleen cells by separation on a Ficoll-Hypaque gradient before testing. Two in vitro lymphoblastoid cell lines, MDCC-LS1 (29) and MDCC-MSB1 (1), developed from MD lymphomas, were maintained at 41°C in RPMI 1640 medium as modified by Hahn et al. (15). Before testing, the cells were centrifuged and washed three times in RPMI 1640.

Antisera. Anti-MATSA serum was prepared in rabbits by four biweekly intravenous injections of viable (6 × 10⁶ to 2 × 10⁷) NYM1 lymphoma cells. Six days after the last injection, serum was collected, heat inactivated, and absorbed three times with a 0.5 volume of washed chicken erythrocytes and four times with a mixture of spleen, thymus, and bursal cells. This serum did not contain anti-MDV antibodies since indirect fluorescent antibody (IFA) tests with MDV-infected primary chicken kidney cells (26) were negative.

Antisera specific for the MHC antigens B₁ and B₂ were produced by allogeneinization with erythrocytes and PBL by using G-B1 and G-B2 recipients which were maintained in isolator units and were free of MDV infection as determined by previously described tests (26). The specificity of these antisera for MHC antigens was verified by hemagglutination tests with blood from birds of known blood group genotypes.

Conjugated antisera that were used were a tetramethylrhodamine isothiocyanate-labeled goat anti-rabbit immunoglobulin G (IgG) (R-GaR) serum (Miles Laboratories, Elkhart, Ind.), a fluorescein isothiocyanate-labeled goat anti-rabbit IgG (F-GaR) serum (GIBCO Laboratories, Grand Island, N.Y.), and a fluorescein isothiocyanate-labeled horse anti-chicken immunoglobulin (F-HaC) serum (Progressive Laboratories, Inc., Washington, D.C.). All antisera were titrated to determine the optimal concentrations required for capping.

Serological tests and capping procedure. IFA tests as described by Witter et al. (34) were used to detect MATSA and MHC antigens. Briefly, 5 × 10⁴ cells suspended in RPMI 1640 were pelleted, suspended in two drops of the appropriate antiserum, and reacted for 30 min at 4°C. The cells were then washed in RPMI 1640, centrifuged, suspended in the appropriate conjugate, and reacted for 20 min at 4°C. After washing in RPMI 1640, the cells were pelleted again and suspended in 1 ml of RPMI 1640 with 10% fetal bovine serum. To induce capping, the cells were incubated in a water bath at 37°C for 1 h. To determine if cocapping of two antigens had occurred, a second IFA procedure with the second antiserum and fluorescent antibody conjugate was performed. After the second IFA procedure, the cells were preserved in 0.1 ml of 50% glycerol in 0.01 M phosphate-buffered saline (pH 7.6). A drop of the cell suspension, spread on a glass slide under a cover slip, was examined with a fluorescent microscope (model 2070; American Optical Corp., Buffalo, N.Y.) equipped with a vertical illuminator. For fluorescein fluorescence, a BG 12 exciter filter, a 550-nm interference filter, and an OG 515 barrier filter were used. For rhodamine fluorescence, an IF 530-nm exciter filter, a 540-nm interference filter, and an OG 570 barrier filter were used. Cells showing fluorescence on less than one-third of the membrane were considered capped. At least 100 cells were observed for each determination. Color photographs were taken with ASA 200 Ektachrome film (Kodak). Exposure times were 30, 60, or 120 s depending upon the intensity of the observed fluorescence.

RESULTS

Tests that were made to determine the specificities of the various antisera are shown in Table 1. The results clearly showed that the antisera used in the capping studies had the desired specificities for MHC antigens and MATSA. One unexpected result was the low percentage of the thymus cells that stained when alloantisera for MHC antigens were used. Only 18% of the G-B1 thymus cells and 15% of the G-B2 thymus cells showed membrane fluorescence after incubation with anti-B₁ and anti-B₂ sera, respectively. This was in marked contrast to the high percentage of spleen cells and PBL that showed specific staining with these sera.

Titrations of antisera revealed that capping was optimum when dilutions of anti-MATSA at 1:10, anti-B₁ at 1:64, and anti-B₂ at 1:8 were used. Best results were obtained when the fluorescent antibody conjugates were diluted at 1:32. Capping of MHC antigens occurred on thymus cells, spleen cells, and PBL from uninfected chickens (Table 2). Capping of MHC antigens on G-B1
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spleen cells is shown in Fig. 1.

MATSA could be capped on cells of the two in vitro lymphoblastoid cell lines MSB1 (Fig. 2a) and LS1 (Fig. 2b). Capping of MATSA occurred on about 50% of the NYM1 lymphoma cells. Approximately 50% of these cells also showed capping of MHC antigens. However, capping of MHC antigens on the transplantable lymphoma cells did not cause capping of MATSA. Figure 3a is a double exposure of an NYM1 cell showing capped MHC antigens and ring staining with the anti-MATSA serum. Similarly, capping of MATSA on NYM1 lymphoma cells did not induce cocapping of MHC antigens (Fig. 3b).

Results of the capping and cocapping attempts are shown in Table 3. Specificity and completeness of the MHC antigen capping reaction were demonstrated when the second IFA procedure was performed with the same anti-B1 and F-HaC sera used in the capping procedure. Cells that were capped did not show ring staining after the

TABLE 1. Specificities of antisera

<table>
<thead>
<tr>
<th>Cells</th>
<th>Antiserum</th>
<th>FA conjugate</th>
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<tr>
<td>G-B1 lymphoma</td>
<td>B1</td>
<td>F-HaC 100</td>
</tr>
<tr>
<td></td>
<td>MATSA</td>
<td>F-GaR 95</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>F-HaC 0</td>
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<tr>
<td>G-B1 thymus</td>
<td>B1</td>
<td>F-HaC 18</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>F-HaC 0</td>
</tr>
<tr>
<td>G-B2 thymus</td>
<td>B1</td>
<td>F-HaC 0</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>F-HaC 15</td>
</tr>
<tr>
<td></td>
<td>MATSA</td>
<td>R-GaR 0</td>
</tr>
<tr>
<td>G-B1 spleen</td>
<td>B1</td>
<td>F-HaC 100</td>
</tr>
<tr>
<td></td>
<td>B2</td>
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<tr>
<td></td>
<td>MATSA</td>
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<tr>
<td>G-B2 spleen</td>
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<tr>
<td></td>
<td>MATSA</td>
<td>R-GaR 0</td>
</tr>
<tr>
<td>G-B1 PBL</td>
<td>B1</td>
<td>F-HaC 100</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>F-HaC 0</td>
</tr>
</tbody>
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* FA, Fluorescent antibody.
* Mean counts for two different observations.

TABLE 2. Capping of chicken MHC antigens on cells from uninfected chickens

<table>
<thead>
<tr>
<th>MHC genotype</th>
<th>Cells</th>
<th>Capping performed with:</th>
<th>Stained cells (%)*</th>
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<tbody>
<tr>
<td></td>
<td>Anti-</td>
<td>FA conjugate*</td>
<td>With caps</td>
</tr>
<tr>
<td></td>
<td>serum</td>
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<tr>
<td></td>
<td>to:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B'/B' thymus</td>
<td>B1</td>
<td>F-HaC 67</td>
<td>67</td>
</tr>
<tr>
<td>spleen</td>
<td>B1</td>
<td>F-HaC 62</td>
<td>62</td>
</tr>
<tr>
<td>PBL</td>
<td>B1</td>
<td>F-HaC 52</td>
<td>52</td>
</tr>
<tr>
<td>B'/B' thymus</td>
<td>B2</td>
<td>F-HaC 65</td>
<td>65</td>
</tr>
<tr>
<td>spleen</td>
<td>B2</td>
<td>F-HaC 69</td>
<td>69</td>
</tr>
</tbody>
</table>

* Mean counts for two different observations.
* FA, Fluorescent antibody.

FIG. 1. Capping of surface MHC antigens on normal chicken spleen cells. Cells were stained at 4°C and capped at 37°C for 1 h as described in the text.
FIG. 2. Capping of MATSA on the surface of MDV-induced lymphoblastoid cell lines. (a) In vitro cell line MDCC-MSBI; (b) in vitro cell line MDCC-LSI.

FIG. 3. Capping of surface antigens on MDCT-NYMI lymphoma cells. Cells were capped and restained at 4°C as described in the text. The field was shifted between the first and second exposures. (a) Double exposure of same cell after capping with anti-B, and F-HaC sera (right) and then retaining at 4°C with anti-MATSA and R-GaR sera (left). (b) Double exposure of same cell after capping with anti-MATSA and R-GaR sera (right) and then retaining at 4°C with anti-B, and F-HaC sera (left).

second IFA procedure, indicating that most, if not all, of the MHC antigens had been capped. The second IFA test for cells capped with anti-MATSA and R-GaR sera was performed with anti-MATSA and F-GaR sera. Staining with the F-GaR serum occurred only on areas which were originally stained with R-GaR serum. These results indicated that the majority of the MATSA antigens had also been capped. Therefore, the independent capping of MATSA and MHC antigens could not be attributed to incompleteness of the capping reaction with either anti-MATSA or anti-B, serum.

DISCUSSION

The low percentage of thymus cells which showed fluorescent staining after incubation with anti-B, or anti-B, serum indicates that there may be a large population of chicken thymus cells which do not possess MHC antigens on their surface. Since the thymus cells that stained showed about the same intensity of fluorescence as spleen cells and PBL, it would appear that the low percentage of positive thymus cells was not due to quantitative differences in MHC antigens on the cell membrane. Of the cells that showed fluorescent staining, capping of MHC antigens occurred as well on thymus cells as on spleen cells or PBL.

Since cocapping of MHC antigens and MATSA did not occur with MDV-induced lymphoma cells, it appears that these antigens are independent cell surface structures. Other studies, in which alloantisera to MHC antigens was found to be incapable of blocking fluorescent staining of anti-MATSA serum (unpublished data and K. K. Murthy, personal communication), also suggest independence of these antigens. These results are in accordance with studies using cells derived from Burkitt lymphoma, in which the Epstein-Barr herpesvirus is thought to be the etiological agent. Fluorescence blocking and cross-blocking tests indicated that Epstein-Barr herpesvirus-determined membrane antigens are sterically distinct from MHC leukocyte antigens on the surface of Epstein-Barr
herpesvirus-carrying lymphoblastoid cell lines (13). Thus, on both MDV and Epstein-Barr herpesvirus-transformed cells, MHC antigens are apparently not modified in a manner analogous to the "altered self" proposed for virus-infected cells (35). The negative cocapping results also suggest that MATSA is not a modified histocompatibility antigen as previously postulated (6).

MATSA may be a virally-determined membrane antigen and a marker for transformation of lymphocytes by MDV but not a tumor-specific antigen. MATSA has been observed on lymphocytes from birds infected with nononcogenic MDV and the herpesvirus of turkeys (21, 22, 27), which is used as a vaccine for MD. Murthy and Calnek (19) found MATSA-bearing cells appearing in the spleen by 5 days after infection with MDV. These authors reported that MATSA was specifically associated with lymphocytes from MDV-infected birds since MATSA was not detected on normal embryonal spleen cells, cells infected with Rous sarcoma virus or T-strain reticuloendotheliosis virus, or on TLT-1 cells, a lymphoblastoid cell line established from Olson's transmissible lymphoid tumor. Studies by Sharma (27) led him to conclude that a cell-mediated immune response to MATSA may be important in vaccine protection against MD. However, Schat and Murthy (22a) found that papain digestion to remove MATSA from the cell surface of MD lymphoblastoid cell lines did not influence the results of an in vitro chromium release assay for cell-mediated immunity. Thus, the specific antigen on MDV-induced lymphoma cells which is recognized by cytotoxic T lymphocytes has not been identified with certainty.

Further studies are required to determine the mechanism by which MHC genes influence susceptibility to MDV and whether there may be histocompatibility restriction associated with the cell-mediated response to MD lymphoma cells. The development of additional transplantable MDV-induced lymphomas in inbred lines of chickens having different MHC genotypes will greatly enhance the possibilities of providing relevant information in this area.

### ACKNOWLEDGMENT

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### LITERATURE CITED

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