Immune Response to Oncornaviruses and Tumor-associated Antigens in the Chicken

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Summary

Two principal virus-directed antigens have been identified on the surface of oncornavirus-infected chick embryo cells. One is identical with the major virus type-specific envelope antigen, which is a glycoprotein with a molecular weight of 85,000. The 2nd antigen (tumor-specific cell surface antigen) is specific for transformed cells, i.e., absent from productively infected but nontransformed cells. This antigen has been identified as a glycoprotein with a molecular weight of 100,000 and was not found in the mature virion. Remarkably, both antigens induce humoral as well as cellular immunity in the chicken. It could be shown that cells can be killed in cytotoxic assays via either the tumor-specific cell surface antigen or the virus envelope glycoprotein alone.

Introduction

Much interest has focused very recently on the immune response, under conditions of nonexperimental infection, to RNA tumor virus-induced antigens, especially to virus structural proteins (e.g., Refs. 6 and 10). Extensive studies have been performed in mammalian systems, but not in the avian systems. In the chicken system, on the other hand, a series of investigations has recently been performed in our laboratory on the immune response to antigens that are induced after experimental infection with ATV. In particular, those antigens that are located at the cell surface and that might act as transplantation-type antigens have been investigated. For technical reasons, most of that work involved sarcoma viruses; however, some evidence indicates that the results obtained herewith might also be valid for leukosis-lymphomatosis viruses.

Earlier in vivo transplantation experiments by several authors had indicated the existence of TSTA in RSV-induced mammalian tumors (reviewed in Ref. 1). By immunization via this TSTA the animals could be protected against a tumor cell challenge. The absence of virus particles in such tumors and of neutralizing antibodies of the tumor-bearing animals suggested that the TSTA were not identical with the Ve antigens, which were later identified as being 2 glycoproteins weighing 85,000 daltons (gp85) and 37,000 daltons (gp37), respectively (4).

In vivo experiments in chickens with the use of the replication-defective Bryan strain of RSV, however, were interpreted to indicate that in the natural host the Ve antigen acted as the main, if not the only, transplantation antigen (20, 21). Later and more recent experiments by Meyers et al. (18), on the other hand, clearly demonstrated that chickens could be immunized by ALV of a subgroup different from that of the RSV used for challenge, i.e., the Ve antigens were of different type specificity. In other words, in these studies, the immunity could not have been caused by the Ve antigen, which at the surface of the cell or the intact virus does not seem to expose a group-specific antigenic determinant (8). This was indicative of a further virus-specific antigen acting as TSTA.

We investigated the TSTA question more thoroughly by a variety of immunological in vitro techniques. The basis for this work was the operational distinction between sarcoma viruses (ASV), which transform CEC, and leukosis viruses (ALV), which replicate in but do not transform CEC. Furthermore, in various subgroups, A through D, both ALV and ASV are available, a fact that we hoped would enable us to distinguish at least between Ve antigen and any further virus-directed antigen being expressed at the tumor cell surface. Sera and spleen lymphocytes from chickens given injections of ALV or ASV were investigated by a variety of methods for their reaction with CEC infected by ALV or transformed by ASV. It was the assumption that these cells in vitro would reflect the antigenic makeup of cells infected or transformed in vivo.

Materials and Methods

Cells and Viruses. CEC used for ALV infection and ASV transformation were prepared from C/O chicken (7, 13). The viruses were as follows. ALV: RAV-1, RAV-3, and NC-1 of Subgroups A, MAV-2 of Subgroup B, NC-D of Subgroup D;
ASV: SR-RSV-1 and PR-RSV of Subgroup A, SR-RSV of Subgroup D, and B77 of Subgroup C (13, 19).

**Sera and Spleen Lymphocytes.** For preparation of immune sera, chickens were given 1 i.v. injection of living virus and were bled 4 to 6 weeks later. For purposes of immune lymphocyte stimulation, chickens were given i.m. injections at least 3 to 4 times at 4-week intervals before the spleens were removed.

**Detection Assays for Cell Surface Antigens.** The immunological methods used were the ferritin-labeled hybrid antibody technique in combination with electron microscopy, immunofluorescence and cytotoxicity tests with humoral antibody, and the radioimmune assay, as well as cellular cytotoxicity tests, all of which have been described in detail previously (8, 13–15). The biochemical analyses of the antigens were by indirect immune precipitation of radioactively labeled material followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (19).

**Results**

**Detection of Cell Surface Antigens by the Immunoferritin Technique.** Electron microscopic studies with ferritin-labeled hybrid antibody indicated a good correlation between the neutralizing capacity of a serum and its antibody staining of the cell surface and of mature budding virus particles (8). Since this reaction was restricted to viruses within the same subgroup it was concluded that the antigen involved was the Ve antigen gp85 (Fig. 1 a and b). From this it followed that the gp85 was also expressed at nonbudding sites of the cell surface. Furthermore, this and more recent studies in our laboratory (R. R. Friis, J. Rohrschneider, and H. Ogura, manuscripts in preparation) indicated that at the budding site the gp85 molecules penetrate the cell surface only with the concomitant assembly of the virus core. It is likely, however, that gp85, as expressed at the nonbudding sites, is not due mainly to adsorption from the outside of degraded virus substructures but rather reflects a true insertion of gp85. This assumption is supported by the finding of Hanafusa et al. (9) that chf (+) cells, which do not synthesize intact virus particles, express Ve antigen at the cell surface.

Antisera prepared against sarcoma virus, especially high-titer neutralizing antisera, showed a most interesting phenomenon in that they also stained cells that were transformed by ASV of a different subgroup (Table 1; Fig 1, c and d). Since this reaction was not found on nontransformed cells productively infected by ALV of a different subgroup, we concluded that a TSSA was involved.

In a parallel study the same pattern as in Table 1 was found for ALV and ASV antisera by the immunofluorescence technique (13).

**Immune Lymphocyte Cytotoxicity via the Ve Antigen and/or TSSA.** Of high interest was the investigation of spleen lymphocytes from ALV- and ASV-infected chickens in a microcytotoxicity test (13). On ALV-infected, i.e., nontransformed, CEC, both lymphocytes (against ALV or ASV) exerted a subgroup-specific cytotoxic effect that was probably due to the Ve antigen. When ASV-transformed CEC were used, however, both kinds of immune lymphocytes reacted group specifically (Table 2), suggesting that the antigen in question was TSSA.

The finding that ALV-induced immune lymphocytes in contrast to humoral antibodies also detected group-specific TSSA was attributed to the fact that lymphocytes were taken after several virus booster injections, i.e., after a longer incubation period that probably was sufficient for the ALV to transform cells of the hematopoietic and lymphatic system, with the consequence of TSSA expression and induction of immune lymphocytes (13). A cross-reaction between ALV- and ASV-induced TSSA was also suggested by in vivo experiments in the chicken (18) and also in the mouse system (2), where animals could be protected against sarcoma challenge by pretreatment with ALV of a different subgroup. More recently, this question has also been tested in vitro and it was found that TSSA-specific chicken or mouse antibody prepared against Rous sarcoma cells specifically reacted with leukemic cells transformed by the avian myeloblastosis virus (16).

**Isolation and Biochemical Analysis of TSSA.** In order to test the usefulness of gp85 and TSSA for *in vivo* immunization, it is most desirable to have these materials in a purified preparation. Having previously isolated and purified the gp85 of these viruses (4, 24), we recently likewise attempted to isolate TSSA. For this purpose, we have developed a sensitive indirect immune precipitation method which has allowed the detection of TSSA in detergent lysate prepared from ASV-transformed CEC that had been labeled with radioactive fucose or glucosamine (3, 19).

By analysis in polyacrylamide gel electrophoresis, radioactively labeled and immune-precipitated TSSA has been

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**Table 1**

<table>
<thead>
<tr>
<th>Chicken antibody prepared against</th>
<th>Infecting viruses</th>
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<tr>
<td>ALV-A</td>
<td>+</td>
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<tr>
<td>ASV-A</td>
<td>+</td>
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<tr>
<td>ALV-D</td>
<td>0</td>
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<td>ASV-D</td>
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* The antigen reacting with the ALV-infected cells is Ve antigen.

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**Table 2**

<table>
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<th>Immune lymphocytes prepared against</th>
<th>Infecting viruses</th>
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<tr>
<td>ALV-A</td>
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<tr>
<td>ALV-D</td>
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<td>ASV-D</td>
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* In contrast to the ALV antisera (see Table 1), immune lymphocytes obtained after several ALV injections also detect TSSA.
identified as a glycoprotein with a molecular weight of 100,000; this is compared with the gp85 and gp37 antigens in Chart 1. Its partial purification has been accomplished by adsorption and elution from a lectin chromatography column, and its isolation on a large scale and in the absence of gp85 should be possible in the near future by using cells transformed by the gp85-defective Bryan strain of RSV that do not reveal the gp85 antigen (L. Rohrschneider, R. R. Friis, M. Hayami, G. Pauli, and H. Bauer, in press).

Detection of Embryonic Antigens. The reexpression of fetal or embryonic antigen has been described in various tumor systems (5). By using antisera from chickens after injection of chicken embryo extracts, such antigens can also be detected on the surface of ASV-transformed CEC. By absorption experiments 1 antigen was detected (EA\textsubscript{T}) that was not identical with TSSA or a virion antigen and which was absent from normal or productively (ALV) infected CEC (16). On the basis of cytotoxicity tests the EA\textsubscript{T} must be considered as a weak antigen. Interestingly, an antigen cross-reacting with EA\textsubscript{T} was found on RSV-transformed mouse cells.

Discussion

From the above reviewed results it appears that at least 2 virus-directed antigens exist at the surface of oncornavirus-transformed cells: the Ve antigen, which is type or subgroup specific; and the TSSA. The latter is assumed to be identical with the TSTA as involved in transplantation experiments in the mouse, since we have shown an immunological cross-reaction between RSV-transformed mouse, hamster, and chicken cells (7, 14) and because the TSTA in mammalian Rous sarcomata does not appear to be Ve antigen. Regarding the Ve antigen, the minor component gp37 was suggested to constitute the spike which at the virus envelope is underneath the outer knobs (gp85) (4). It is, therefore, not clear whether only the major and outer component gp85 or also gp37 is accessible to immunoreagents at the intact cell surface.

The demonstration of humoral and cellular immunity against tumor cells in the ATV system is well in agreement with the general concept of "immune surveillance" of the immune system of the host against arising tumor cells, i.e., the chicken is very probably capable of eliminating single tumor cells or small tumors. It is still unclear, however, which of the new antigens described above, and whether humoral or cellular immunity, plays the most important role in the immune defense. From the work of Hayami et al. (11, 12) one can deduce that it may be the cellular immune response that plays the dominant role in the tumor rejection response.

In vitro experiments reviewed above have shown clearly that Ve antigens, TSSA, and EA\textsubscript{T} are expressed on the cell surface in sufficient amounts to mediate the lymphocytic response (13, 14). The observation on the poor immunogenicity of chicken tumors induced by the Ve antigen-defective Bryan strain of RSV (20, 21) also suggests that immunity against Ve antigen might play an important role in immune surveillance.

The 3rd kind of tumor cell surface antigen, namely that of embryonic origin, once seemed a promising tool for the induction of antitumor immunity. However, it is now becoming increasingly evident that such an approach has severe limitations. Immunization does not always lead to detectable tumor protection (23); on the contrary, even immunostimulation of tumor growth has been reported (reviewed in Ref. 16).

The formation of ATV tumor-specific cytotoxic lymphocytes has clearly been demonstrated in the chicken (13, 22), as well as in Japanese quail (11, 12) and in mammals (14). Immune lymphocytes killed cells via the TSSA as well as via the Ve antigens (13). While the cytotoxic effect of mammalian antibody directed against ATV-induced TSSA was clearly demonstrated in vitro (14), this has not been tested.

Chart 1. Isolation of TSSA. Either normal, virus-transformed, or nontransformed productively infected CEC (chf-) were labeled with \textsuperscript{3}H)fucose (100 \(\mu\)Ci/ml) for 24 hr, washed on ice, and extracted for 15 min with cold lysis buffer (0.02 M Tris-HCl, pH 7.5-0.05 M NaCl, 0.5% NP 40). The supernatant was centrifuged at high speed (100,000 \(\times\) g, 60 min) after addition of deoxycholate to 0.5%. Aliquots were then caused to react with various chicken immune IgG and precipitated with rabbit anti-chicken IgG. The latter reaction reflects a group-specific determinant of gp85, which has been described elsewhere (Ref. 3; Rohrschneider et al., Virology, 67: 234-241, 1975).

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\textsuperscript{3}H)fucose-labeled ASV-A virus was electrophoresed in a parallel gel (---) or normal chicken IgG (----). The latter reaction reflects a group-specific determinant of gp85, which has been described elsewhere (Ref. 3; Rohrschneider et al., Virology, 67: 234-241, 1975).
\end{tabular}
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for the respective avian immunoglobulins, because chicken immunoglobulins do not activate mammalian complement, and chicken complement is very unstable.

For investigation of whether humoral antibodies or immune lymphocytes predominate in vivo in the immunological defense of tumors, birds have the advantage over mammals in that they not only have a distinct thymus, controlling cellular immunity, but also a distinct bursa fabricii, controlling humoral immunity. This provides a unique model for manipulating the thymus-dependent cellular as well as the bursa-dependent humoral immune response.

In extensive studies on Japanese quails either bearing ASV tumors or after tumor regression, Hayami et al. (11, 12) demonstrated that humoral serum factors obtained from birds with progressively growing tumors blocked in vitro the cytotoxic effect of lymphocytes against tumor cells. From the various experimental approaches it became obvious that the blocking factors represented mainly IgG or IgG-antigen complexes and rarely soluble tumor cell antigen. Although suggestive, it is not conclusive from these data that immunoglobulins in this system have only a blocking but no cytotoxic function in vivo, because a cytotoxic activity requiring complement, as available in vivo, has never been tested in vitro by these authors. Thus, a cytotoxic in vivo effect of humoral IgG is still possible and conceivable.

The immunosurveillance of tumors is probably impaired by the phenomenon of “blocking” or “enhancing” antibody which seem mainly to represent soluble antigen-antibody complexes preventing the cytotoxic humoral antibody from attaching to the tumor cell or blocking the cytotoxic action of immune lymphocytes. At present, nothing is known about the mechanisms that favor this unwelcome in vivo phenomenon. In a system like the one described here one could ask, for example, whether enhancing effects are due to TSSA or rather to the Ve antigen. This could probably be studied by immunization with the respective soluble antigens. The avian system, at present, appears to be the most advantageous system for investigating such relevant problems. Not only can the humoral antibody (bursa fabricii) and the immune lymphocyte (thymus) factories be selectively eliminated, and thus the relevance of humoral or cell-bound immunity be investigated, but procedures for isolating the 2 antigens have been developed. While the Ve antigen can be isolated from purified virus preparation (4, 24), the TSSA has now been isolated from tumor cells by detergent treatment and has been partially purified (3, 19).

With these facilities at hand, it is our purpose to investigate the role of Ve antigen and of TSSA in the immune reaction of the host against tumor cells and the possible usefulness of these 2 kinds of antigens for prophylaxis against tumors.

References

Fig. 1. Thin sections of CEC infected or transformed by ALV and ASV, respectively, after staining with ferritin-labeled hybrid antibody. a, Subgroup A leukemia virus-infected cells stained with homologous antiserum; b, the same cells as in a stained with antiserum against leukemia virus of a heterologous subgroup (anti-ALV-B); c, Subgroup D sarcoma virus-transformed cells stained with homologous antiserum; d, the same cells as in c stained with antiserum against sarcoma virus of a heterologous subgroup (anti-ASV-A).
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