Evidence that Avian Tumour Virus-immune Chicken Sera Recognize Only Viral Structural Antigens on the Surface of Avian Tumour Virus-infected Cells

By LOUIS F. QUALTIERE*† AND PAUL MEYERS‡

Department of Microbiology, Mayo Clinic Foundation and Mayo Medical School, Rochester, Minnesota 55901, U.S.A.

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SUMMARY

The specificity of the humoral response of chickens to avian tumour viruses (ATV) was investigated by reacting ATV-immune sera with Triton X-100 extracts of uninfected, infected and transformed chicken embryo fibroblasts. Analysis of these immune reactions by polyacrylamide gel electrophoresis revealed that avian leukosis virus-challenged and Rous sarcoma virus-challenged chickens recognized only two major cell surface antigens of 100000 and 29000 mol. wt. which were present on transformed and non-transformed virus-producing cells. No labelled antigens were precipitated from uninfected cells or transformed cells producing the envelope-defective mutant RSV(−). The antigens were shown to be related to the major envelope glycoproteins of the virus and to contain group-specific determinants common to ATV subgroups B and C. No group-specific determinants common to ATV subgroups A and B or subgroups A and C were detected. Chickens were found to have a strong antibody response to the 100000 and 29000 mol. wt. proteins prior to and during tumour rejection, even in the absence of neutralizing antibody to the challenge virus. No tumour-specific surface antigen distinct from the virion structural antigens was detected by any of the immune chicken sera on any of the transformed cells tested.

INTRODUCTION

The definition of avian tumour virus (ATV)-associated antigens, as recognized by the immune system of ATV-infected and/or tumour-bearing chickens, has been affected by shifting emphases over the last few decades. Immunological reaction to tumours in Rous sarcoma virus (RSV)-infected chickens, hence determining the fate of the tumour, was initially believed to be directed toward those glycoprotein structural components of the envelope of the virus (Vogt & Ishizaki, 1965) now known as gp85 and gp35 (sometimes called gp37) and collectively called viral envelope antigens (VEA). Subsequently, however, experiments, performed primarily on mammals, pointed to the existence of a response directed against another tumour antigen, distinct from the virus structural antigens but common to all members of the various RSV subgroups and apparently virus-coded (Koldovsky, 1969; Bauer, 1974; Wainberg & Phillips, 1976). This antigen, variously called tumour-associated transplantation antigen, tumour-specific surface antigen (TSSA) or virus-induced cell-surface antigen (VISA, CSA) was believed to be of major importance in eliciting an effective immune response to RSV-induced tumours, with the cellular response being paramount in tumour rejection (Bauer, 1974; Wainberg & Phillips, 1976; Kurth et al., 1979). Subsequently, however, a number of workers using a variety of techniques have been unable to define immunologically or to isolate tumour antigens distinct from VEA,
using sera from RSV-infected birds (Phillips & Perdue, 1976, 1979; Morris & Fritz, 1976; Kurth et al., 1976). Others found that both humoral and cellular responses to putative non-virion, tumour-associated antigens were weak or non-existent (Meyers & Qualtiere, 1977; Hall et al., 1979) especially after a single primary immunization with RSV (Hall et al., 1979). Because of this, the claimed immunochemical definition of the TSSA (Rohrschneider et al., 1975a) was both withdrawn (Bauer, 1978) and disputed (Phillips & Perdue, 1979).

In addition, evidence emerged which indicated that group-specific antigenicities were present on the gp85 moiety of various subgroups of RSV, as recognized by rabbit (Rohrschneider et al., 1975b; Brugge et al., 1978, 1979) and chicken (Halpern & Friis, 1978) antisera, thus further confusing the interpretation of some previous results.

We have demonstrated by direct binding assays that humoral reactivity to ATV-infected cells can be found in RSV tumour-bearing birds' sera both in the presence and, more importantly, in the absence of antiviral neutralizing antibody (Meyers & Qualtiere, 1977; Hall et al., 1979). It is the purpose of this report to define the specificity of this humoral response in terms of antigen recognition and furthermore, to determine if a TSSA, distinct from viral structural antigens, is indeed recognized by ATV-infected chickens. Experiments using heterologous systems, while yielding extremely valuable information, do not necessarily indicate the validity of antigenic recognition in the 'natural' host [e.g. the RSV src gene product pp60-src is immunogenic in a variety of mammals, but apparently not in chickens (Collett et al., 1979; Brugge et al., 1978, 1979) unless massive and repeated doses of virus are administered (Ellwart-Tschurtz & Kurth, 1981)]. Therefore, we have characterized the humoral immune recognition patterns and the specificity of a large number of chicken immune sera, using well-characterized viruses and cells and a highly sensitive radioimmune precipitation (RIP) assay.

**METHODS**

_Viruses and virus assays._ All viruses were from cloned stocks and were grown on chick helper factor (chf)-negative chick embryo fibroblasts (CEF). Avian leukosis viruses (ALV) were assayed by a radioimmunoassay (RIA) after growth on CEF (Meyers, 1976), while RSV was titrated by a standard focus formation assay (Meyers & Dougerty, 1971). Focus-reduction assays for the detection of neutralizing antibody were performed as described (Meyers & Dougerty, 1971). The viruses used in this study were ALV-F42 of ATV subgroup A, its antigenically homologous transforming RSV pseudotype RSV(F42), Schmidt-Ruppin strain RSV of subgroup B (SR-RSV-B), Prague strain RSV of subgroup C (PR-RSV-C) and a spontaneous transformation-defective mutant of SR-RSV-B, designated td R02-2-SR-B whose isolation has been described (Hall et al., 1979). This latter virus will subsequently be referred to as R02.

_Cells._ CEF of the C/E genotype were grown from single White Leghorn embryos (SPAFAS, Norwich, Conn., U.S.A.) as described by Dougerty et al. (1963), and were chf-negative and ATV group-specific (gs) antigen-negative when tested, according to previously published criteria (Meyers & Qualtiere, 1977). CEF transformed by, and producing the envelope-defective RSV (−) were produced by established methods (Hanafusa et al., 1970; Kawai & Hanafusa, 1973) and will be referred to below as R(−)C cells. Infected CEF to be used for radiolabelling were produced by plating of RSV or ALV at high multiplicities (>10) followed by two or three cell passages. In the case of RSV-infected CEF, the cells were used when at least 80 to 85% transformed as judged by microscopic examination. All cells were grown in Dulbecco's modified Eagle's medium with sodium pyruvate, supplemented with 10% calf serum (Gibco) and all were at passage level five or six when used.

_Antisera._ Chicken blood was obtained by venipuncture, the blood was allowed to clot overnight, and the resulting serum was heat-inactivated at 56 °C for 30 min. All sera, including some which had been used in previous studies (Meyers & Qualtiere, 1977; Hall et al., 1979), were then stored at -20 °C until needed.

_Antisera._ Chick- and gs-negative White Leghorn chickens and eggs from a flock free of Marek's disease virus, exogenous ALV and common avian pathogens were purchased from SPAFAS, Inc. Birds were inoculated with virus between 3 and 5 weeks of age, except in the case of ALV-F42 congenitally infected animals which were infected as embryos by previously described (Meyers, 1976).

_Cell surface labelling and solubilization._ Cell surface sialoglycoproteins were 3H-labelled with sodium borohydride by the method described by Gahmberg & Andersson (1977), using mild periodate treatment at 0 'C with short incubation times, followed by reduction with NaB₃H₄, to selectively label external sialic acid-containing glycoproteins. This method affects surface glycoprotein labelling patterns similar to those obtained with the neuraminidase-galactose oxidase technique (Gahmberg & Andersson, 1977). Uninfected CEF and virus-infected CEF were removed by rapid pipetting from the surfaces of 100 mm cell culture plates and were washed by three consecutive centrifugations (500 g, 10 min) with 0.01 m-phosphate-buffered saline, 0.1 m-NaCl, pH 7.2 (PBS) at
Recognition of ATV antigens by chickens

Table 1. Characteristics of virus-infected CEF used

<table>
<thead>
<tr>
<th>CEF infected with*</th>
<th>Antigenic subgroup of infecting virus</th>
<th>Transformed phenotype</th>
<th>Productive infection (VEA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>NA†</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SR-RSV-B</td>
<td>B</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R02</td>
<td>B</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>ALV-F42</td>
<td>A</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>RSV(F42)</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RSV(-)</td>
<td>NA</td>
<td>+</td>
<td>- ‡</td>
</tr>
</tbody>
</table>

* All cells were derived from the same single embryo.
† NA, Not applicable.
‡ Since RSV(-) is a mutant that does not express the envelope surface glycoproteins, antigens (VEA) are not detectable and the virus itself is non-infectious except under special conditions.

4 °C. After the last wash, the cell pellet (1 × 10^6 cells) was chilled to 0 °C in an ice-bath and then mildly oxidized by resuspension in 1 ml of 1 mM-sodium periodate in PBS at 0 °C. After 10 min, the reaction was terminated by the addition of 0.3 ml of 0.1 M-glycerol in PBS and the cells were then washed with PBS by three centrifugations, as before at 0 °C. The final cell pellet was then resuspended at room temperature in 1 ml PBS containing 2 mCi NaB^3H_4 (Research Products International, Elk Grove Village, Ill., U.S.A.), which had been dissolved in 0.1 M-sodium hydroxide immediately before use. The cells were incubated for 30 min at room temperature and the labelling was then terminated by the addition of 5 ml PBS. The cells were then centrifuged as before and washed twice more with PBS. Infected or uninfected labelled CEF were mixed with 1% Triton X-100 in PBS containing 2 mM-phenylmethylsulphonyl fluoride (PMSF; Pierce Chemical Company, Rockford, Ill., U.S.A.) at a ratio of 1 ml Triton solution per 2 × 10^7 cells. After 30 min at 25 °C, nuclei and particulate matter were removed by centrifugation at 10000 g for 1 h. The supernatant fluid was then decanted and exhaustively dialysed against 0.1% Triton X-100 in PBS for 2 days. The same types of test cells were also labelled by a second method using [3H]-glucosamine. Cells growing in 100 mm tissue culture plates were metabolically labelled by replacing the old medium with 8.5 ml of glucose-free medium containing 0.266 mCi of tritiated glucosamine (New England Nuclear) and 1% foetal calf serum which had been dialysed exhaustively against PBS. Cells were then incubated for 36 h at 37 °C, washed five times with 3 ml portions of warm PBS, solubilized directly in the dish with 1% Triton X-100 and treated as described above for NaB^3H_4 labelled solubilized cells.

**Immune precipitation of solubilized membranes.** For immune precipitation, 0-1 to 0.2 ml samples of the solubilized cells containing 1 × 10^6 to 0.5 × 10^6 ct/min of [3H]-glucosamine-labelled extract, or 2 × 10^4 to 5 × 10^4 ct/min of NaB^3H_4-labelled extract, were added to 0.1 ml of chicken antisera (1:20 dilution) and the mixtures incubated overnight at 4 °C. The chicken IgG was then bound by immune precipitation with 0.1 ml of titrated rabbit anti-chicken IgG. The resultant precipitate was centrifuged at 3500 g for 30 min, washed three times with 0.1% Triton X-100 in PBS and then dissolved in 2% SDS containing 2-mercaptoethanol before analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

**Polyacrylamide gel electrophoresis.** The SDS-PAGE, which was previously described in detail (Qualtiere & Pearson, 1979), used the discontinuous buffer system of Laemmli (1970) with a stacking gel of 3% (w/v) acrylamide (1.2 cm high) and as the running gel an exponential gradient of 10 to 15% (w/v) acrylamide (11.0 cm long). The samples were electrophoresed for 18 h at 4 °C at a constant 60 V and the gel was then vacuum-dried in a Hoeffer slab gel dryer. The gels were processed by the fluorographic technique of Bonner & Laskey (1974). The dried gel was then exposed to Royal Omat X-ray film (Kodak) for 2 to 20 days at -70 °C. The 125I-labelled proteins which were used as molecular weight standards were myosin (210000), β-galactosidase (130000), human serum albumin (69000), ovalbumin (43000), soybean trypsin inhibitor (21500) and lysozyme (12500).

**RESULTS**

**Immune precipitation of tritiated sodium borohydride-labelled avian tumour virus cell surface proteins**

In order to differentiate humoral reactivity to the various ATV cell surface proteins present on ATV-infected and/or transformed cells, immune and pre-immune sera from chickens infected with SR-RSV-B were incubated with the Triton X-100 solubilized NaB^3H_4-labelled cell extracts of normal CEF and with five different ATV-infected and/or transformed CEF selected to give a representation of the entire range of the various known or putative tumour and/or viral antigens (Table 1).
Table 2. Chicken antisera used

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>Bird no.</th>
<th>Virus injected*</th>
<th>Serum taken at time after RSV injection (days)</th>
<th>Neutralizing antibody (N.I.)†</th>
<th>Antibody to SR-B VEA</th>
<th>Antibody to RIP§</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1539</td>
<td>None</td>
<td>60</td>
<td>2·0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1580</td>
<td>SR-RSV-B</td>
<td>20</td>
<td>1·3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>1580</td>
<td></td>
<td>60</td>
<td>&gt;178·5</td>
<td>NT†</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>1581</td>
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<td>0</td>
<td>1·6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>1581</td>
<td>SR-RSV-B</td>
<td>20</td>
<td>1·3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>1581</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>7</td>
<td>1582</td>
<td>SR-RSV-B</td>
<td>60</td>
<td>&gt;178·5</td>
<td>NT†</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>1586</td>
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<td>9</td>
<td>1586</td>
<td>SR-RSV-B</td>
<td>20</td>
<td>1·2</td>
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<td>+</td>
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<tr>
<td>10</td>
<td>1586</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>842</td>
<td>SR-RSV-B</td>
<td>33</td>
<td>2·6</td>
<td>NT†</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>841</td>
<td>SR-RSV-B</td>
<td>33</td>
<td>&gt;276·0</td>
<td>NT†</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>722</td>
<td>SR-RSV-B¶</td>
<td>33</td>
<td>&gt;353·0</td>
<td>NT†</td>
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<td>SR-RSV-B¶</td>
<td>33</td>
<td>4·1</td>
<td>NT†</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>904</td>
<td>PR-RSV-C</td>
<td>33</td>
<td>2·2</td>
<td>NT†</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>900</td>
<td>PR-RSV-C</td>
<td>33</td>
<td>18·8</td>
<td>NT†</td>
<td>+</td>
</tr>
</tbody>
</table>

* All birds were injected with 100 f.f.u. of the indicated virus.
† Neutralization index against homologous RSV. A N.I. < 5·0 is considered negative (Meyers & Dougherty, 1971).
‡ As judged by criteria outlined in Hall et al. (1979).
§ As judged by precipitation of the 100000 and/or 29000 mol. wt. polypeptides.
∥ NT, Not tested.
¶ Infected as embryos with ALV-F42 and injected with SR-RSV-B at 3 weeks post-hatch.

Five SR-RSV-B immune sera with high neutralizing antibody (Table 2, serum numbers 3, 6, 7, 10 and 12) collected at 33 or 60 days post-inoculation were tested using the Triton X-100 cell extracts. A representative autoradiograph of the SDS–PAGE findings is shown in Fig. 1. None of these five different SR-RSV-B immune sera precipitated any detectable polypeptides from the Triton X-100 extracts of R(−)C cells, normal CEF, or ALV-F42- and RSV(F42)-infected cells (Fig. 1, lanes 2, 3, 4 and 8). Only polypeptides of 100000 and 29000 molecular weight were precipitated from R02-infected and SR-RSV-B-transformed cell extracts (Fig. 1, lanes 7 and 9) and only by immune sera, since none of the corresponding pre-immune sera from the same birds (not listed in Table 2) reacted with these latter extracts (Fig. 1, lanes 5 and 6).

The molecular weights of the two cell surface proteins precipitated from subgroup B virus-producing cells by SR-RSV-B immune chicken sera correspond with the known molecular weights of the two major viral envelope structural glycoproteins of the ATV group. When the same antisera that precipitated the 100000 and 29000 molecular weight proteins from the cell extracts were reacted with sucrose gradient-purified, Triton X-100-solubilized SR-RSV-B virions, they precipitated the major virion glycoproteins, gp85 and gp35, at 88000 and 32000 mol. wt. respectively (Fig. 1, lane 10). This demonstrates that the 100000 and 29000 mol. wt. antigens on the surface of infected cells share common determinants with the major virion glycoproteins, gp85 and gp35. The somewhat lower apparent molecular weights of the envelope antigens on free, extracellular virus are consistent with extracellular cleavage of the precursor Pr92env immediately after the budding process (Klemenz & Diggelmann, 1979; also see below).

In previous studies (Meyers & Qualtiere, 1977; Hall et al., 1979) it was reported that, shortly after virus infection, some SR-RSV-B-infected chickens developed humoral reactivity to SR-RSV-B cell surface antigens, as detected by an isotopic antiglobulin test (IAT), despite the absence of detectable neutralizing antibody to the virus. Some of these birds eventually developed this antibody, while some did not, even though in both cases their tumours regressed. Therefore, it was of interest to determine what antigens chickens recognized on SR-RSV-B-transformed cells, before development of neutralizing antibody, since this early humoral
Recognition of ATV antigens by chickens

Fig. 1. Fluorograph of the SDS-PAGE analysis of the ATV-surface proteins precipitated by a SR-RSV-B neutralizing antiserum, from the periodate–sodium borohydride-labelled Triton X-100 extracts of cells. Lane 2, R(−)C cells; lane 3, normal CEF; lane 4, ALV-F42-infected CEF; lane 7, R02-infected CEF; lane 8, RSV(F42)-transformed CEF; lane 9, SR-RSV-B-transformed CEF. Lanes 5 and 6 show pre-immune serum reacted with the Triton X-100 extracts of R02-infected CEF and SR-RSV-B-transformed cells respectively. Lane 1, 12SI-labelled mol. wt. marker proteins: myosin, 210000; β-galactosidase, 130000; human serum albumin, 69000; ovalbumin, 43000; soybean trypsin inhibitor, 21500; lysozyme, 12500. Lane 10 contains the [3H]glucosamine-labelled polypeptides precipitated from Triton X-100-solubilized, gradient-purified SR-RSV-B by a SR-RSV-B-neutralizing antiserum on a separate gel run. The mol. wt. standards for this run (lane 11) were: myosin, 210000; phosphorylase B, 92500; human serum albumin, 69000; ovalbumin, 43000; carbonic anhydrase, 30000; lactoglobulin H, 18300.

immune response might detect antigens involved in tumour rejection. To this end, four chicken sera (Table 2, sera 2, 5, 9 and 11) lacking detectable neutralizing antibody, but demonstrating antibody reactive with SR-RSV-B-transformed cells when tested using an IAT (Hall et al., 1979), were incubated with the 3H-labelled, Triton X-100-solubilized cell extracts of the six different indicator cell types. Somewhat surprisingly, analysis by SDS–PAGE of the immune precipitates demonstrated that these sera had the same specificities as those chicken sera that did contain neutralizing antibody to the homologous infecting virus (SR-RSV-B). As can be seen in Fig. 2, an autoradiograph of the immune precipitate patterns of one of these sera (the four had identical profiles), shows that the only detectable reactivity was to 100000 and 29000 mol. wt. polypeptides precipitated from extracts of SR-RSV-B-transformed cells and R02-infected cells. No labelled antigen was precipitated from extracts of cells infected and/or transformed with viruses of a different subgroup [ALV-F42 or RSV(F42)] or from normal CEF or R(−)C-transformed cells. It is clear that the birds from which these sera were taken were mounting a humoral immune response to the structural VEA of the infecting virus even though the humoral antibody response was negative when judged by a biological method, the very sensitive focus reduction assay for neutralizing antibody. Thus, there is a discordance in the results obtained using RIP and neutralization assays.

Earlier work (Meyers & Quatliere, 1977; Quatliere & Meyers, 1979) established that chickens that are congenitally infected with ALV-F42 and are humorally tolerant to this virus are able to respond, in some cases, with neutralizing antibody to a subsequent challenge of virus of a heterologous subgroup B RSV. These patterns of humoral response are present against a background of a highly impaired ability of these animals to control tumour growth when challenged with RSV of subgroups A, B or C. For example, when ALV-F42 congenitally infected birds are challenged with doses between $10^2$ and $10^4$ focus-forming units of SR-RSV-B, many birds respond with neutralizing antibody to the challenge virus, but most fail to cause the
induced tumour to regress. Because of these observations, which indicated a gross failure of the immune system of such birds to recognize tumour antigens in the absence of any general impairment of humoral or cellular immunity (Meyers & Dougherty, 1971; Meyers et al., 1976), it also seemed appropriate to identify the antigens that are recognized humorally by ALV-F42 congenitally infected chickens after challenge with a heterologous subgroup of avian sarcoma virus. RIP analysis of sera (numbers 13 and 14; Table 2) from these SR-RSV-B-challenged, ALV-F42 congenitally infected birds, with (no. 13) and without (no. 14) detectable neutralizing antibody to SR-RSV-B, showed precipitation of the 100000 and 29000 mol. wt. antigens only from solubilized, SR-RSV-B- or R02-infected cell extracts and no significant precipitation of any polypeptide from the other cell extracts (data not shown). Confirming our earlier conclusions of the existence of a true humoral tolerance in ALV-F42 congenitally infected chickens (Qualtiere & Meyers, 1979), no labelled polypeptides were precipitated from ALV-F42 or RSV(F42) cell extracts by any of the sera from congenitally infected birds. Challenge of subgroup A congenitally infected birds with subgroup B (SR-RSV) or C (PR-RSV) avian sarcoma virus also does not 'break' tolerance to the subgroup A VEA, since subgroup A VEA were not precipitated in the RIP using sera from birds so challenged (data not shown).

Several authors have reported the presence of group-specific determinants of the VEA (Halpern & Friis, 1978; Schlesinger, 1976; Rohrschneider et al., 1975b). We note here that we were unable to detect any group-specific reactivity between subgroup A and subgroup B VEA as shown by the inability of any of the SR-RSV-B antisera to precipitate any detectable labelled polypeptides from the ALV-F42- or RSV(F42)-infected cell extracts (Fig. 1 to 3) and by the failure of high titred chicken anti-ALV-F42 antisera to precipitate corresponding VEA from the SR-RSV-B or R02 extracts (data not shown). We were, however, able to demonstrate group-
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Fig. 3. Fluorograph of the SDS–PAGE analysis of the [3H]borohydride-labelled ATV surface proteins. Lanes 1 to 6, precipitated by an immune non-neutralizing antiserum of a chicken challenged with PR-RSV-C, from the Triton X-100 extracts of cells. Lane 1, R(−)C cells; lane 2, normal CEF; lane 3, ALV-F42-infected CEF; lane 4, R02-infected CEF; lane 5, RSV(F42)-transformed CEF; lane 6, SR-RSV-B-transformed CEF. Lanes 8 to 10, precipitated by a PR-RSV-C-neutralizing antiserum from an ALV-F42 congenitally infected chicken challenged with PR-RSV-C, from the Triton X-100 extracts of cells. Lane 8, R02-infected CEF; lane 9, RSV(F42)-transformed CEF; lane 10, SR-RSV-B-transformed CEF. Lane 7 contains the 125I-labelled standards (see Fig. 1, lane 1 for molecular weights).

specific reactivity between VEA of subgroups B and C. In Fig. 3, both non-congenitally infected and ALV-F42 congenitally infected birds, when challenged with PR-RSV-C, responded with antisera capable of reacting with SR-RSV-B and R02 VEA (Fig. 3, lanes 4, 6, 8 and 10) but not to subgroup A VEA (Fig. 3, lanes 3, 5 and 9). This group-specific reactivity can be demonstrated in serum from PR-RSV-C-infected birds regardless of whether the serum does (lanes 8 to 10) or does not (lanes 1 to 6) contain detectable neutralizing antibody to PR-RSV-C. As before, no polypeptides were precipitated from the R(−)C extracts nor could any additional antigens be identified in the other transformed cell extracts (Fig. 3, lanes 1, 4, 5, 9 and 10).

Immune precipitation of [3H]glucosamine-labelled avian tumour virus membrane antigens

In order to determine if any other ATV cell-surface proteins were present in ATV-infected and/or -transformed cells, but were not labelled by the highly specific periodate-[3H]-borohydride labelling, the same types of indicator cells as listed in Table 1 were metabolically labelled using [3H]glucosamine. The cells were then subjected to analysis for ATV-specific proteins by the RIP technique. A representative autoradiograph of one of the ten SR-RSV-B immune antisera tested is shown in Fig. 4. In agreement with the results of the periodate-borohydride labelling, [3H]glucosamine-labelled polypeptides of 100000 and 27000 mol. wt. were consistently precipitated from the Triton X-100 extract of the SR-RSV-B-infected cells (Fig. 4, lane 6). In addition, some minor bands at 72000 and 40000 mol. wt. could occasionally be detected. However, several additional bands could consistently be precipitated from the Triton X-100 extract of the R02-infected cells, at 72000, 51000, 40000 and 12500 mol. wt., in
addition to the major virion glycoprotein at 100,000 mol. wt. (Fig. 4, lane 4). The 27,000 mol. wt. component was never precipitated from the R02 extract. No major glycoproteins were precipitated from the R(−) cells, normal CEF, or from the ALV-F42- or RSV(F42)-infected cells (Fig. 4, lanes 1, 2, 3, and 5), confirming the previous RIP analysis of the periodate–borohydride labelling. The pre-injection sera, as before, precipitated no labelled polypeptides (Fig. 4, lanes 8 and 9). These results were reproducible with both neutralizing and non-neutralizing antisera and also with sera from ALV-F42 congenitally infected chickens after challenge with SR-RSV-B or PR-RSV-C. Apart from the above-mentioned exceptions, mainly with R02-infected cell extracts, the data obtained from the RIP analysis of the [3H]glucosamine-labelled cell extract were consistent with that generated by the RIP analysis of the periodate–borohydride-labelled cell extracts.

Finally, because of the possibility that there might be important ATV-induced or synthesized proteins which might not be labelled by the two mainly glycoprotein-specific labelling procedures just described, the same group of indicator cells were surface-labelled by the lactoperoxidase method (Qualtiere & Pearson, 1979), washed, extracted with Triton X-100 and subjected to analysis for ATV-specific proteins by the RIP technique. Although a somewhat larger number of non-specifically precipitated labelled polypeptides appeared on the autoradiographs, perhaps masking some specifically precipitated, the results completely substantiated the findings detailed above for the periodate–borohydride and [3H]glucosamine labelling protocols and hence these fluorographs are not shown.
Recognition of ATV antigens by chickens

DISCUSSION

First, we have shown that the specificity of chicken neutralizing sera to SR-RSV-B was directed towards two major cell surface proteins of 100000 and 29000 mol. wt. The spread of the higher mol. wt. (100000) band, which extends over the mol. wt. range of 87000 to 110000, is not surprising as this band is very probably composed of precursor (gPr92env) molecules in various stages of glycosylation as well as mature VEA (Buchhagen & Hanafusa, 1978; England et al., 1977; Klemenz & Diggelmann, 1979). This seems especially likely since we were dealing with virus-infected cells (which probably have virus in various stages of maturation on the cell surface), rather than mature free virus particles. The lower mol. wt. band at 29000 may represent specific precipitation by serum antibody of this component but it is more likely to be coprecipitating with the 100000 mol. wt. antigen as does gp37 with gp85 (Rohrschneider et al., 1975b). The two labelled antigens were only precipitated from Triton X-100-solubilized extracts of ATV subgroup B-infected cells, regardless of whether the cells were transformed (SR-RSV-B) or not (R02). No labelled polypeptides were precipitated from normal CEF by immune sera, demonstrating that the antigens were virus-specific. No labelled cell surface polypeptides were precipitated from the R(−)C cell extracts, indicating that these transformed cells, which produce an envelope-defective virus, have no glycoprotein VEA and also lack transformation-specific antigen. These observations are further substantiated by the absence of any proteins precipitated from the SR-RSV-B-transformed cells other than VEA. We were unable to detect group-specific determinants common to ATV subgroups A and B VEA since none of the hyper-immune subgroup B antisera precipitated labelled polypeptides from the two subgroup A-infected cell extracts. This observation was not just a property of the reaction of subgroup B immune sera with subgroup A-infected cells, since in reciprocal experiments two high-titred anti-RSV(F42) sera, which precipitated their homologous VEA at 100000 and 29000 mol. wt., did not precipitate subgroup B VEA nor did they detect a common group-specific TSSA present in R(−)C SR-RSV-B- or RSV(F42)-transformed cell extracts (data not shown).

These data argue strongly against any humorally recognizable common or cross-reactive group-specific non-virion TSSA being present on both subgroup A- and subgroup B-transformed cells. As was the case with the neutralizing antisera, non-neutralizing sera collected early after SR-RSV-B virus challenge (10 to 20 days), but showing immune reactivity to RSV-transformed cells in a direct binding assay (Meyers & Qualtiere, 1978; Hall et al., 1979), also precipitated only the homologous VEA at 100000 and 29000 mol. wt. from the SR-RSV-B and R02 cell extracts (Fig. 2). Once again, no evidence of any group-specific TSSA could be found since no polypeptides could be precipitated from transformed R(−)C or RSV(F42) cell extracts. The precipitation of the subgroup B VEA by non-neutralizing sera from birds injected with SR-RSV-B substantiated the findings of the direct binding assay, that there is a significant humoral response to SR-RSV-B VEA determinants prior to and during tumour growth and regression, and before neutralizing antibody is detectable. It follows, then, that the absence of neutralizing antibody to an ATV cannot always be used as a reliable indicator of a lack of early immunity to ATV VEA.

In a previous publication (Meyers & Qualtiere, 1977) we showed that congenitally infected birds, although tolerant to a subgroup A virus, could respond humorally to a subgroup B or C RSV challenge, but that this immune response was unable to curb rapid and progressive growth of PR-RSV-C or SR-RSV-B tumours, which regressed in non-congenitally infected birds. We have now shown by RIP that ALV-F42 congenitally infected birds make no antibody that can react with VEA of subgroup A despite their humoral response to VEA or RSV of subgroups B or C after challenge, and must be making antibody only to unique determinants of subgroup B or C VEA.

Although we have been unable to demonstrate any degree of cross-reactivity between subgroup A and B VEA by RIP analysis, there are clearly group-specific determinants common to the subgroup B and C VEA (Fig. 3) since both normal and ALV-F42 congenitally infected chickens, when challenged with PR-RSV-C, responded with both neutralizing and non-neutralizing antibody to the subgroup B 100000 and 29000 mol. wt. antigens. Thus our data

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confirm in one respect and contradict in another the observations of those who have found

group-specific non-neutralizing antibody to the VEA of subgroups A, B and C viruses. These

studies, using either rabbit antiserum (Schlesinger, 1976; Rohrschneider et al., 1975b) or serum

from infected chickens (Halpern & Friis, 1978; Rohrschneider et al., 1975b) showed cross-

reactive group-specific determinants between subgroups A, B or C. These findings differ from

ours since we found that our chicken sera did not recognize a group-specific determinant

common to both subgroups A and B. In the case of rabbit antisera, this anomaly may be simply

due to the ability of a xenogenic species to recognize determinants different to those recognized

in the chicken. In addition, the strains of virus used by us and other workers differ. The use of

ATV-immune chicken sera produced by multiple RSV injections may also result in immunity

primarily toward the carbohydrate moiety of gp85 and broader reactivity of antisera. Specificity

of cross-reactivities of gp85 from various ATV may depend, in part, on the carbohydrate moiety

gp85 and the antisera used as well as the assay method (Brugge et al., 1978; Van Eldik et al.,

1978; Collins et al., 1978).

One minor point which requires some explanation is the presence of the additional

glucosamine-labelled bands at 72000, 51000, 40000 and 12500 mol. wt. which were precipitated

from the R02 extracts. These remain unidentified, but may represent aberrant cleavage products

of the gPr92env precursors or other differences in processing, or increased lability of glycosylated

proteins, perhaps related to the fact that R02 is a spontaneous td mutant, having a deletion in all

or some large portion of the src region, which is adjacent to the env region of the viral genome. It

even possible that R02 may contain a deletion extending into the 3' end of the env gene.

In summary then, our results support the views of Phillips & Purdue (1976, 1979) who also

failed to detect an avian tumour-specific antigen, using two selected chicken sera under highly

controlled conditions with an entirely different labelling procedure. Of course, one explanation

for the failure of RIP analysis to detect a putative TSSA could be the specificity of the periodate-

borohydride 3H-labelling, which requires the antigen to contain accessible sialic acid residues.

However, this probably is not an acceptable explanation since Triton X-100 extracts of the same

indicator cells metabolically labelled with [3H]glucosamine also detected only VEA in

productively transformed cell extracts (Fig. 4). Most importantly, it seems unlikely that our

inability to detect a unique ATV-induced non-virion TSSA can be attributed to failure to label

adequately any such putative antigen. That a TSSA does exist but was not solubilized under the

conditions used is also a possibility. Barring this latter possibility, if a unique polypeptide(s)

containing TSSA properties does exist, then it may be mainly, or only, recognized by the T-cell

components of the chicken's immune system or it is present at such low concentrations as to be

impossible to detect, even by the highly sensitive RIP assay. More to the point, in the absence of

any compelling new data to the contrary, there would appear to be no need to invoke the

existence of an ATV-induced TSSA, distinct from virion-coded structural antigens or their

precursors, at least in terms of its humoral recognition by the immune system of the chicken.

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