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Autologous Immune Responses to the Major Oncornavirus Polypeptides in Unmanipulated AKR/J Mice[1]

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The autologous immune response of AKR/J mice to the structural proteins of murine leukemia virus (MuLV) was examined. Immunoglobulins from the renal glomeruli were chemically eluted, separated from antigens, recovered, and tested for immunological reactivity against MuLV structural proteins. Analyzing immune precipitates obtained after mixing radiolabeled Tween-disrupted MuLV preparations with eluates from AKR/J mice on sodium dodecyl sulfate-gel electrophoresis, we found evidence of antibodies to the major classes of MuLV structural components: gp70, gp45, p30, and one or more proteins in the 10,000- to 15,000-dalton class. Using rate zonal centrifugation we confirmed that the eluates from AKR/J glomeruli contained antibody(s) that bound specifically to p30. These results indicate that AKR/J mice spontaneously mount immune responses against the major oncornavirus polypeptide antigens.

As embryos, AKR/J mice receive a generalized infection of murine leukemia virus (MuLV) imparted by vertical transmission from their mothers (6). After birth, each animal maintains virus in blood and organs throughout life (4). Initially thought to be immunologically incapable of making an immune response to the virus (15), in fact such mice are competent in mounting antiviral immune responses (1, 5, 13, 18, 20). We previously observed that 6- to 10-month-old AKR/J mice developed high incidences of glomerulonephritis owing to the trapping of immune complexes, presumably viral-antiviral antibody complexes, in the renal glomeruli (15). Immunoglobulins (Ig's) eluted from AKR/J renal glomeruli were tentatively identified as antibody(s) to MuLV by three approaches: first, they immunospecifically stained the surfaces of C57Bl/6 leukemia cells induced by passage A Gross leukemia virus; second, they stained the cytoplasm of several cells from AKR thymus tissue (15); and third, they blocked RNA-dependent DNA reverse transcriptase activity (5). Others have found evidence for natural antibodies to oncornavirus in the blood of AKR/J mice (1, 13).

Although the evidence at hand clearly indicates that such mice can mount immune responses against MuLV, the viral polypeptides to which antibody(s) is being produced, complexed, and deposited in the renal glomeruli remains unknown. Is the antibody(s) response against all known viral subcomponents or against only some? To answer this question, we obtained Ig that had been bound to the glomeruli of AKR/J mice, formed immune precipitates with radiolabeled MuLV proteins, and analyzed the participants in the reaction by using sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis and rate zonal centrifugation.

MATERIALS AND METHODS

Preparation of kidneys. AKR/J mice of both sexes were obtained from Jackson Laboratories, Bar Harbor, Me. Random testing indicated that the mice were free of lymphocytic choriomeningitis virus, lactic dehydrogenase virus, and polyoma virus infections. The kidneys of mice sacrificed at 7 to 10 months of age were washed in cold phosphate-buffered saline and then snap-frozen in liquid nitrogen. With a cryostat, 4-μm thin sections were cut, fixed in ether alcohol and then 95% alcohol, and examined by direct fluorescence microscopy for the presence of host Ig. Details as to handling of these tissues, preparation and specificity of antisera, and its conjugation to fluorescein isothiocyanate have been published (18, 20). Kidneys with glomeruli that showed moderate to heavy deposits of Ig were pooled for subsequent immunochemical treatment.

Elution. Fifty to one hundred kidneys were used for each preparation. Kidneys were homogenized, washed with PBS-EDTA, and eluted with glycine-hydrochloride buffer (0.02 M, pH 2.7) for 45 min at 37 C as previously detailed (18). After low-speed centrifugation (700 $\times$ g) to clear debris, the supernatant was concentrated with a membrane filter (300XM, Amicon) under 40 to 60 mm Hg of pressure, resulting in two fractions: the filtrate, containing molecules smaller than 100,000 daltons, and the concentrate, containing those molecules predominantly

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larger than 100,000 daltons. The concentrate was placed on a 5 to 20% linear sucrose gradient (wt/vol in STE [0.1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4]) and centrifuged in a SW41 rotor at 200,000 × g for 18 h. Multiple 12-drop fractions were obtained from each tube.

Virus. Three types of MuLV were used in this study. MuLV (Gross) was purified from K36 cultured cells by isopycnic centrifugation; MuLV (Scripps) was purified as described earlier (3); MuLV (AKR) was obtained from J. Gruber, Special Virus Cancer Program, National Cancer Institute, Bethesda, Md. Virus preparations were used when they came off the gradient or frozen at −80 C until use. Virus was disrupted with 1% Tween, and approximately 50 to 100 μg of viral protein was mixed with 2 mCi of ^{125}I and 50 μg of chloramine T. After 5 min of incubation on ice, 50 μg of sodium metabisulphite and 10 μl of a 1% KI solution were added. Highly purified p30 MuLV (Scripps) obtained from J. Gruber, Special Virus Cancer Program, and murine Ig prepared by Pevikon block electrophoresis (11) were radiolabeled with ^{125}I or ^{131}I and served as markers for velocity gradient analysis. Human immunoglobulin G (IgG) immunoglobulin M and (IgM) were also radioiodinated with ^{131}I and served as markers for SDS gels.

Antisera. Rabbit antiserum to mouse Ig was prepared as described elsewhere (17). At an antigen concentration of 10 mg/ml, the antiserum was immunochemically pure by both Ouchterlony and immunoelectrophoretic analyses. Goat antiserum to mouse Ig was the gift of R. Levy (Scripps Clinic and Research Foundation). We used amounts of these antisera sufficient to precipitate over 99% of the mouse Ig in the reaction mixture.

Immune precipitation. Indirect immune precipitations were carried out as described earlier (3). Test tubes were coated with 50 μl of normal rabbit or goat serum and 50 μl of 5% Nonidet P-40. (Normal rabbit serum was used for rabbit antimouse IgG precipitates and normal goat serum was used for goat anti-mouse IgG precipitates.) Five microliters of serum from normal 4- to 6-week-old BALB/c mice, alone or mixed with the eluate and 0.5 ml of STE, were added to the coated tubes, followed by the addition of ^{125}I-labeled antigen. Primary binding was allowed to proceed for 15 min at 37 C. Sufficient anti-mouse IgG was then added to precipitate over 99% of the Ig from the primary reaction mixture. After 15 min of incubation at 37 C, immune precipitates were collected by centrifugation at 2,000 × g for 15 min, washed with STE containing 0.1% Nonidet P-40, and then with ice-cold distilled water.

Polyacrylamide disc gel electrophoresis. Immune precipitates were solubilized in 8 M urea, 1% SDS, and 2% β-mercaptoethanol, and then ^{131}I-labeled human IgG and IgM were added as molecular-size markers. This mixture was then heated to 56 C for 30 min, then to 100 C for 2 min, before electrophoresis. Samples were analyzed by electrophoresis on 6% acrylamide gels prepared as described by Weber and Osborn (24). Electrophoresis was for 2.5 h at a constant current of 15 mA per gel. Gels were frozen on dry ice, and then cut into 1-mm slices with a Joyce Loebl gel slicer (Joyce, Loebl & Co., Inc., Burlington, Mass.) and counted in a Nuclear-Chicago automatic gamma counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Control gels containing Ig markers with viral preparation alone or with a precipitate formed by the viral preparation, normal mouse serum, and antimouse Ig were run concurrently. Data were corrected for background, ^{131}I crossover, counting efficiency, and were plotted with a Hewlett-Packard system (Hewlett-Packard Co., Palo Alto, Calif.).

Rate zonal centrifugation. For analysis by rate zonal centrifugation, 20 μl of ^{125}I-labeled p30 (10 ng; specific activity, 20 to 60 μCi/μg) was mixed with 10 or 20 μl of AKR eluate (150 μg Ig/ml). After 15 min of incubation at 37 C with gentle agitation, 100 μl of rabbit anti-mouse Ig was added for an additional 15-min incubation. Other samples contained 20 μl of ^{125}I-labeled p30 alone, or 20 μl of ^{125}I-labeled p30 with 20 μl of AKR eluate. The samples were layered on top of a 5 to 20% linear sucrose gradient in STE, and centrifuged for 20 h at 114,000 × g in a 50.1 rotor. The gradients were tapped from the top, and the 12-drop samples collected in individual tubes were counted for the presence of ^{125}I radioactivity.

RESULTS

Purification of eluted Ig separated from MuLV p30. In initial experiments using conventional low-acid and low-molar buffers (18), we were unable to demonstrate antibody to p30 in the Ig eluted from glomeruli of AKR/J mice, despite finding p30 antigen in the same region of the glomerulus as Ig by immunofluorescence (M. B. A. Oldstone, unpublished observations). This was probably caused by the simultaneous collection of large amounts of viral antigens along with the eluted Ig. Determinations of amounts of p30 in the AKR/J eluted material indicated that after neutralization such preparations had 264 to 335 ng of p30 for each 25 mg of kidney protein. To separate the lg eluted from the glomeruli from p30 and other viral proteins, we first passed the eluate through a 300XM Amicon filter, which allowed most of the lower-molecular-weight proteins (antigens: <100,000 daltons) to pass through and retained the larger proteins (>100,000 daltons). The concentrate was then placed on a 5 to 20% linear sucrose gradient and centrifuged. In preliminary experiments, this method efficiently separated bovine serum albumin from antibody to bovine serum albumin obtained from immune precipitates while retaining the immunological reactivity of each. As seen in Fig. 1, Ig was efficiently separated from p30 with this technique.

Demonstration of antibody(s) to oncornavirus polypeptides in the eluates from the renal glomeruli of AKR/J mice. The specificity of the Ig eluted from the glomeruli of AKR/J mice to oncornaviral polypeptides was determined by indirect immune precipitation. After washing,

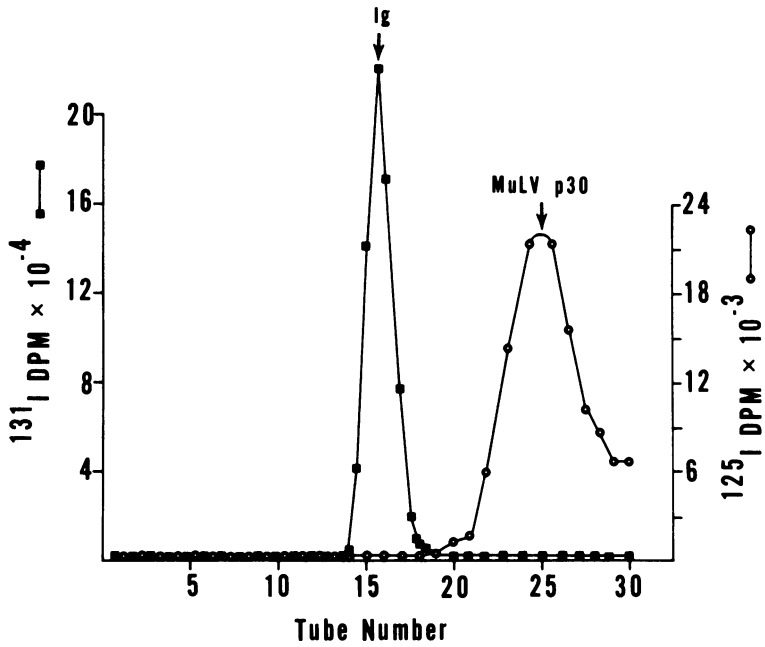

FIG. 1. *Separation of Ig from MuLV p30. Purified murine IgG was mixed with MuLV p30, then centrifuged at 200,000 × g for 18 h through a 5 to 20% linear sucrose gradient. Fractions were collected and the amount of ^{125}I and ^{131}I was determined. Symbols: (■) ^{131}I-labeled murine IgG; (○) ^{125}I-labeled MuLV p30.*

these precipitates contained from 1.1 to 2.4% of the labeled MuLV proteins. In contrast, trapping controls had 0.5 to 0.8% of the labeled proteins. The antigens against which the antibodies in the eluate were reacting were identified by SDS-polyacrylamide gel electrophoresis. Antibodies to gp70, gp45, p30, and p10-15 MuLV (Gross) and MuLV (AKR) were found (Fig. 2). These antibodies also reacted with an FMR type MuLV (Scripps) with a pattern similar to that seen with AKR and Gross virus, except for a more prominent gp70 peak in the MuLV (Scripps) (Fig. 3). This is most likely related to the higher gp70 concentrations in the MuLV (Scripps) preparation. Concurrent trapping controls showed that insignificant amounts of labeled viral preparations were precipitated (Fig. 2 and 3).

The presence of antibodies to p30 in eluates from the AKR/J kidneys was confirmed by rate zonal centrifugation studies. As shown in Fig. 4, a significant shift in the position of radiolabeled p30 occurred when the antigen was incubated with AKR/J eluates. The major p30 radioactivity peak previously found at about 72% of the gradient was shifted to the bottom (heavier) part of the gradient after the addition of AKR eluate. When rabbit antimouse Ig was added to the complex formed between ^{125}I-labeled p30 and the AKR eluate, the radiolabeled counts moved completely to the bottom of the gradient (Fig. 5). In contrast, the addition of rabbit anti-mouse Ig to ^{125}I-labeled p30 alone did not result in a shift of the radioactive counts from the expected position of ^{125}I p30.

DISCUSSION

From these results, it is seen that AKR/J mice apparently can mount an immune response against the major polypeptides of oncornavirus. Thus, by SDS-acrylamide gel electrophoresis we showed that eluates from the kidneys of unmanipulated AKR/J mice contained antibodies to gp70, gp45, p30, and one or more proteins of the 10,000- to 15,000-dalton class. Under the conditions used, resolution of viral polypeptides smaller than 15,000 daltons could not be determined. Although antibodies against MuLV (AKR, Gross, and Scripps) polypeptides of gp70, gp45, p30, and p10-15 were found, we noted that greater amounts of gp70 occurred in the immune precipitates from MuLV (Scripps) than were found in the precipitates from MuLV (AKR and Gross). Other experiments confirmed that the AKR/J eluates contained antibody that bound specifically to p30, because after incubation of the eluate Ig with radiolabeled p30, immune complexes formed that could be analyzed by rate zonal centrifugation.

Previous difficulties in demonstrating antibodies to oncornaviral polypeptides in similar

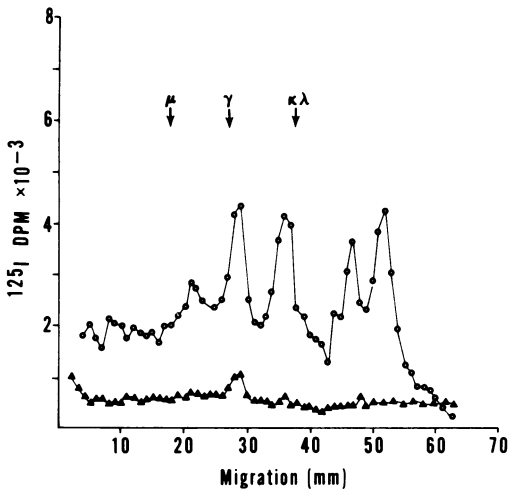

FIG. 2. *SDS-polyacrylamide gel electrophoresis of MuLV (AKR) proteins that react with antibody from AKR kidney eluates. Radioiodinated MuLV (ARK) proteins were incubated with normal BALB/c serum or with normal BALB/c serum mixed with 0.75 μg of Ig isolated from renal glomeruli of AKR/J mice, and then goat antimurine Ig was added to precipitate all the murine Ig in the mixture. After washing, the immune precipitate was suspended in 8 M urea, 2% β-mercaptoethanol, and 1% SDS and heated, then analyzed on 6% polyacrylamide gels. Specific precipitate: ^{125}I-labeled MuLV (AKR) proteins that reacted with antibodies from the AKR renal eluate (○). Trapping control precipitate: ^{125}I-labeled MuLV (AKR) proteins that were found in the normal murine Ig, goat antimurine Ig precipitate (▲). Positions of μ, γ, and Kλ marker proteins are indicated by arrows.*

renal eluates were most likely from technical problems with the elution procedure. At that time, only trace amounts of antibody to p30 were found using a sensitive radioimmune assay, probably because large amounts of free p30 that appeared in the eluted fraction. This suggested that any antiviral antibodies eluted from the renal glomeruli after dissociation of virus-antibody bonds by low-pH, low-molar buffer probably recombined with viral antigens that are in excess after the mixture is restored to normal pH and molarity. To overcome these difficulties, we separated the viral proteins from the antibody during the elution technique by first passing them through an Amicon filter and then by rate zonal centrifugation. This procedure may be of value to others in need of segregating viral antigens from Ig.

Studies in different laboratories with various model systems have clearly indicated that, despite nonfatal infection as a fetus, the individual at birth and later in life can mount an immune response against the viral agents that he carries. Thus, animals persistently infected with lymphocytic choriomeningitis virus (16, 18), lactic dehydrogenase virus (12, 19, 23), oncornaviruses (1, 5, 13, 18, 20), Aleutian disease of mink (21, 22), and equine infectious anemia

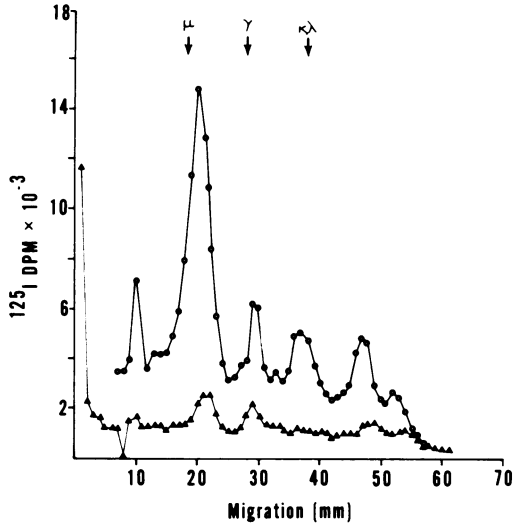

FIG. 3. *SDS-polyacrylamide gel electrophoresis of MuLV (Scripps) proteins that react with antibody from AKR kidney eluates. Radioiodinated MuLV (Scripps) proteins were analyzed as described in Fig. 2. Specific precipitate: ^{125}I-labeled MuLV (Scripps) proteins that reacted with antibodies from the AKR eluate (○). Trapping control precipitate: ^{125}I-labeled MuLV (Scripps) proteins that were found in the normal mouse Ig, goat anti-mouse Ig precipitate (▲). Positions of μ, γ, and Kλ marker proteins are indicated by arrows.*

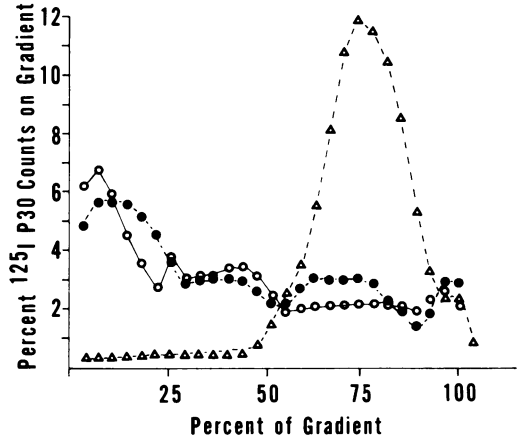

FIG. 4. *Eluates from AKR/J glomeruli bind to MuLV p30. Radioiodinated MuLV p30 alone (△) or mixed with glomerular eluates obtained from two different AKR kidney pools (pool 1 [○], pool 2 [●]) were layered on top of 5 to 20% linear sucrose gradient. After centrifugation for 20 h at 114,000 × g, 12-drop samples were collected in individual tubes and assayed for radioactive counts.*

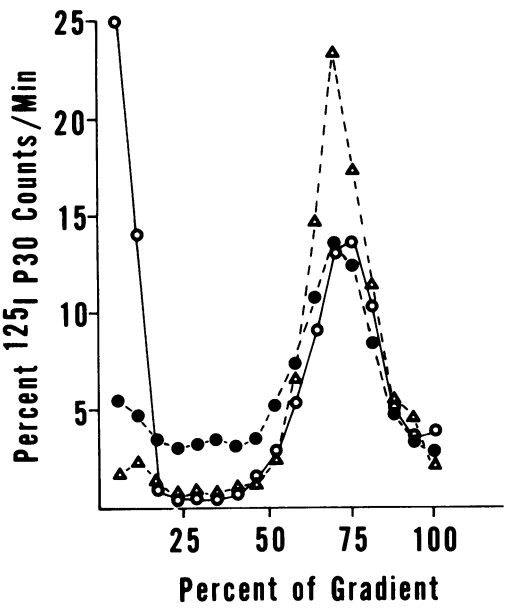

FIG. 5. *Ig in the eluates from AKR/J glomeruli bind to MuLV p30. Radioiodinated MuLV alone (△), mixed with a glomerular eluate (●), or mixed with the glomerular eluate and monospecific antibody to murine Ig (○) were layered on top of a 5 to 20% linear sucrose gradient. After centrifugation for 20 h at 114,000 × g, 12-drop samples were collected in individual tubes and assayed for radioactive counts.*

(2, 10) not only are not immunologically tolerant in that they mount antibody responses against the viruses they carry throughout life, but also develop immune complex deposits in their glomeruli that sometimes result in glomerulonephritis (reviewed in reference 14). The present study indicates that in one model system studied in depth, AKR/J mice naturally infected with MuLV, immune responses are made against all known structures of major oncornavirus polypeptides. Others studying the autogenous humoral immune responses to endogenous RNA tumor virus found different reactivities of IgM and IgG to virus envelope antigens (8). In most of our experiments we used a rabbit anti-mouse Ig serum that bound predominantly with murine IgG but also showed some, though slight, reactivity to murine IgM. Hence, we could not evaluate the differences in IgG or IgM responses against viral antigens. In contrast to previous reports (7, 8), we were able to find antibodies to p30. Results similar to those presented here have been reported in studies of New Zealand mice. In that instance, antibodies reactive with oncornavirus polypeptides were also found deposited in the renal glomeruli (9).

Finally, presumably all the multiple antibody(s) made against oncornavirus polypeptides and pulled off the glomeruli of AKR/J kidney had contributed to the immune complex deposits and any resultant disease. Futher experiments will determine whether there is a difference in phlogogeneity of different complexes and whether immune responses to all viral polypeptides also occur in other persistent viral infections.

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