

PATHOGENESIS OF ARTERITIS OF SL/Ni MICE

Possible Lytic Effect of Anti-gp70 Antibodies on Vascular Smooth Muscle Cells

BY MASAOKI MIYAZAWA, MASATO NOSE, MASAJI KAWASHIMA, AND
MASAHISA KYOGOKU

From the Department of Pathology, Tohoku University School of Medicine, Sendai 980, Japan

Systemic necrotizing arteritis is one of the primary lesions of human autoimmune diseases (1, 2). The most frequently postulated mechanism for the production of necrotizing vasculitis is the deposition of circulating immune complexes (ICs)¹ in vessel walls (3). Experimental studies in animals (4, 5) have established, at least in the case of acute serum sickness, the mechanism for IC localization in tissues and development of vascular injury. However, the factors influencing the deposition of circulating ICs in specific portion of the vasculature remain speculative (3), and our present knowledge about the pathogenesis of vascular injury cannot adequately explain such a divergent spectrum of vasculitides which includes granulomatous inflammation of larger arteries and necrosis of small veins, capillaries, or arterioles of various sizes (2, 6). Moreover, the presence of ICs in the circulation itself is not always associated with vasculitides. For example, several strains of mice predisposed to murine SLE all exhibit high levels of circulating ICs (7). Nevertheless, the development of arteritis is relatively uncommon among them, except in older MRL/Mp-*lpr/lpr* (MRL/1) mice (7, 8). Therefore, it is appropriate to analyze other pathogenetic factors, besides IC-mediated tissue injury, that might mediate the arterial injury seen in human and animal autoimmune diseases.

We report here the first murine model of spontaneously developing necrotizing polyarteritis (NPA) in which antibody-dependent, complement-mediated destruction of arterial smooth muscle cells may play a crucial role in initiating the production of vascular injury.

The model, the inbred SL/Ni strain of mice (H-2^a), was established by Dr. Y.

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¹ *Abbreviations used in this paper:* ABC, avidin-biotinylated horseradish peroxidase complex; H & E, hematoxylin and eosin; HRP, horseradish peroxidase; IC, immune complex; IF, immunofluorescence; MCF, Mink cell focus-inducing; MuLV, murine leukemia virus; NPA, necrotizing polyarteritis; PAP, peroxidase-anti-peroxidase soluble complex; PAS, periodic acid-Schiff; PLP, periodate-lysine-paraformaldehyde; TNE, 0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl, and 1 mM EDTA buffer.

Nishizuka (9), Aichi Cancer Center Research Institute, Nagoya, Japan from his colony of a lymphoma-prone strain of albino mice, SL, in which a decrease in the incidence of lymphoma and spontaneous development of autoimmunity were simultaneously noticed.

Fully developed arterial lesions of SL/Ni mice, which were usually found in the mice older than 9 mo, were histopathologically quite similar to human polyarteritis nodosa, characterized by segmental fibrinoid necrosis of arterial walls with dense perivascular inflammatory cell infiltration. (9–12). Females were affected with a higher frequency than males and almost all multiparous females had arteritis in parametrial tissues and/or ovaries (13). But the most surprising finding was the budding of a large number of murine leukemia viral (MuLV) C-type particles from plasma membrane of smooth muscle cells in the media, which was observed just before or at the onset of arteritis (10, 12). Yoshiki et al. (14) demonstrated the depositions of MuLV gp70, p30, and p15E in affected vascular walls of SL/Ni mice along with mouse IgG, and suggested the possibility that locally produced viral-antiviral ICs might mediate the vascular injury. However, our studies of early arterial lesions revealed that degeneration and necrosis of smooth muscle cells in the media without dense deposition of ICs or infiltration of neutrophils were the earliest detectable changes in affected arteries, and histologic and electron microscopic features of the degenerating smooth muscle cells suggested the possibility of antibody-dependent, complement-mediated cytotoxicity to the vascular component.

To test this hypothesis, we analyzed immunohistochemical and immunoelectron microscopic aspects of the early arterial lesions and also studied virologic and serologic abnormalities of SL/Ni mice.

Materials and Methods

Mice. Original breeding pairs of SL/Ni mice were kindly provided by Dr. Y. Nishizuka and have been bred and maintained in our laboratory. Either 1–3-mo- or 9–15-mo-old female mice were used in this study. BALB/cAJcl mice were purchased from Clea Japan, Inc., Tokyo, Japan, and were used as age- and sex-matched normal controls.

Histopathology. Immediately after mice were killed, major salivary glands, tracheae, thyroid glands, esophagi, lungs, hearts, aortas, livers, spleens, lymph nodes, kidneys, uteri, and ovaries with parametrial tissues were removed and were fixed in 10% buffered formalin. 3- μ m sections from paraffin-embedded tissues were stained with hematoxylin and eosin (H & E), periodic acid-Schiff (PAS), and elastica-Masson trichrome stains.

Immunofluorescence (IF) Studies of Tissues and Cells. Kidneys, ovaries, and parametrial tissues from SL/Ni mice were snap frozen and 4- μ m cryostat sections were stained directly (15) with FITC-conjugated IgG fraction of rabbit anti-mouse IgG and anti-mouse IgM antisera (Miles Laboratories, Inc., Naperville, IL), and FITC-conjugated IgG fraction of goat anti-mouse C3 antiserum (CooperBiomedical, Inc., Malvern, PA). For detection of antiviral natural antibodies, a subconfluent layer of infected or uninfected NIH 3T3 cells grown on glass cover slips was incubated with dilutions of SL/Ni or BALB/c sera, rinsed three times with HBSS, and was stained with the FITC-conjugated rabbit anti-mouse IgG and anti-mouse IgM antibodies. Since natural antibodies to AKR ecotropic MuLV had been found in older BALB/c mice (16) and we could detect anti-A-5 cell antibodies in some older mice of this strain, sera from young (2–3 mo old) BALB/c mice were used as negative controls in further studies. To detect MuLV envelope antigens in tissues, we used an indirect staining with biotinylated mouse mAbs and FITC-conjugated avidin D (Vector Laboratories, Inc., Burlingame, CA).

Electron Microscopy and Immunoelectron Microscopy. For electron microscopy the para-

metrial tissues were fixed in 2% glutaraldehyde, postfixed in 2% osmium tetroxide, dehydrated, and embedded in Epon 821. For immunoelectron microscopic studies, a part of the parametrial tissues was fixed in the periodate-lysine-paraformaldehyde (PLP) fixative and processed according to McLean and Nakane (17). Fab' fragment of the anti-mouse IgG and anti-mouse C3 antibodies was prepared in our laboratory and was labeled with horseradish peroxidase (HRP) (18). A preembedding method (19) was used to stain frozen sections of the PLP-fixed parametrium and bound Fab' was visualized with the diaminobenzidine reaction (20). Ultrathin sections were stained with uranyl acetate and lead nitrate.

Cell Culture. BALB/3T3 clone A-31 cells, Mv1Lu mink lung cells, and S⁺L⁻MiCl₁ mink cells were purchased from Flow Laboratories, Inc., McLean, VA. NIH 3T3 cells and XC cells were kindly provided by Dr. T. Ebina, Department of Bacteriology, Tohoku University School of Medicine. All these cell lines were grown and maintained in DME supplemented either with 5% heat-inactivated calf serum for NIH 3T3 and XC cells or with 10% heat-inactivated FCS for BALB/3T3, Mv1Lu, and MiCl₁ cells.

Titration of MuLV and Establishment of Clones from Virus-infected Fibroblasts. UV-XC plaque assays (21) and S⁺L⁻MiCl₁ focus formation assays (22) were performed for titration of ecotropic and xenotropic viruses in the spleen, respectively, and *Fv-1*-restricted host range specificity of ecotropic viruses was determined by a quantitative XC plaque technique (23). To establish a chronically infected fibroblast line, spleen cells from a 15-month-old SL/Ni female mouse were seeded as infectious centers onto NIH 3T3 cells and a clone of the infected fibroblasts, A-5, which was producing the highest level of ecotropic virus among several XC⁺ clones, was selected by limiting dilution. Reactivity of a panel of anti-MuLV mAbs with the A-5 cells was tested by a published method (24) and culture supernatant of A-5 cells was titrated for ecotropic, xenotropic, and mink cell focus-inducing (MCF) viruses by a fluorescent focus assay using mAbs (25).

Microcytotoxicity Assay. This assay was performed according to a published method (26) using A-5 or uninfected NIH 3T3 cells grown in Terasaki Microtest plates as target cells. Cells in triplicate wells were incubated at 37°C with twofold dilutions of test sera from SL/Ni and BALB/c mice for 1 h, washed, and then incubated at 37°C with murine or Guinea pig complement for 35 min. Control wells received the diluent (DME) instead of serum dilutions. Fresh sera from 2–3-month-old BALB/c mice were prepared as a complement source (27) and were diluted 1:2 according to a previous titration. Lyophilized Guinea pig serum was purchased from Miles Laboratories, Inc. and diluted 1:10. Percent specific cytotoxicity was determined by nigrosine dye exclusion (26) using the following formula: Percent specific cytotoxicity = $100 \times [(\text{percent dead cells in the experimental wells}) - (\text{percent dead cells in the control wells})] / [100 - \text{percent dead cells in the control wells}]$.

Virus Purification. Cell-free supernatant of A-5 cell cultures was collected daily and was concentrated by ultrafiltration. Viral particles were further concentrated by repeated centrifugation into a 15/65% sucrose density interface (28), and separation of virions was accomplished by isopycnic centrifugation in a Percol density gradient (29). After centrifugation at 30,000 *g* for 20 min in an isotonic solution of Percol (Pharmacia Fine Chemicals, Uppsala, Sweden) prepared with the 0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl, and 1 mM EDTA buffer (TNE) containing 0.25 M sucrose, a single visible band was seen near the center of the gradient column. This band was found to possess MuLV infectivity and the virions recovered from the band exhibited typical C-type morphology in electron micrographs.

Monoclonal and Polyclonal Antibodies to MuLV. Hybridoma lines 24-8 and 24-9 (24) and mAbs 372 (30) and R187 (31) were kindly provided by Drs. John L. Portis and Bruce Chesebro, Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories (RML), Hamilton, MO, and goat anti-Rauscher MuLV gp70 antiserum was kindly supplied by Dr. Hans Wigzell, Department of Immunology, Karolinska Institutet, Stockholm, Sweden. For IF studies antibodies were purified by ammonium sulfate precipitation and affinity chromatography using protein A-conjugated Sepharose CL-4B (Pharmacia Fine Chemicals) (32) and were labeled with biotin (33).

TABLE I
Organ Distribution of NPA in Older SL/Ni Mice

Organ	Mice ex- amined	Mice with NPA	
	<i>n</i>	<i>n</i>	%
Parametrium and/or ovary	49	22	45
Major salivary glands	54	19	35
Pancreas	51	3	6
Liver	51	1	2
Kidney	51	7	14
Paratracheal tissue	51	6	12
Lung	51	4	8
Aorta and/or coronary	51	3	6
Lymph node	51	2	4

Western Blot Immunoassays. Western blotting analysis of viral proteins was performed basically according to the original method (34). The purified viral particles were directly diluted into SDS sample buffers and were lysed either in the presence or in the absence of 50 mM dithiothreitol. Blotted proteins were detected by an enzyme-linked antibody method, instead of the original radiolabeled protein A technique, using biotinylated horse anti-mouse IgG antibody and the avidin-biotinylated HRP complex (ABC) reagent (Vector Laboratories, Inc.) with the HRP Color Developing Reagent (Bio-Rad Laboratories, Richmond, CA) as the substrate. Rat mAb R187 and the anti-Rauscher gp70 goat antibody were detected by using biotinylated rabbit anti-rat IgG antibody (Vector Laboratories, Inc.) and the ABC reagent, or HRP-conjugated anti-goat Ig rabbit antibody and rabbit peroxidase-anti-peroxidase soluble complex (PAP) (Dako Corp., Copenhagen, Denmark), respectively.

Each band on nitrocellulose paper representing structural proteins of the purified ecotropic virus was identified by using mAb 24-8 specific for the disulfide-linked heterodimer of murine leukemia viral gp70 and p15E (gp85) anti-p30 mAb R187, anti-p15E mAb 372, and the anti-Rauscher gp70 antiserum. The overall electrophoretic pattern of the structural proteins of the SL/Ni ecotropic virus was similar to that of other MuLVs. The intensity of fractions detected as bands was graded on a score of - to ++.

Results

Histopathology and Incidence of Fully Developed NPA in Older SL/Ni Mice. 54 female SL/Ni mice at the age of 9-14 mo were examined to determine the incidence and organ distribution of NPA. Almost all of these mice were affected by severe mesangial or membranoproliferative glomerulonephritis. The distribution of NPA by organ is shown in Table I. NPA was most frequently found in the parametrium (the uterine arteries) and/or the ovary, and major salivary glands were affected with a comparable frequency. The cumulative incidence of NPA in the 54 older SL/Ni mice was 50%. Histopathologically, the NPA in an advanced stage was characterized by segmental fibrinoid necrosis of the media with encircling dense inflammatory cell infiltration (Fig. 1A). The integrity of the internal and external elastic laminae was interrupted and the vessel wall was disrupted with dense infiltration of neutrophils and mononuclear cells (Fig. 1B). In some instances, the media was completely replaced by fibrinoid material and the vascular lumen was narrowed with multiplication of the elastic laminae (Fig. 1C). In IF studies of the uterine and ovarian arteries at the comparable stage of

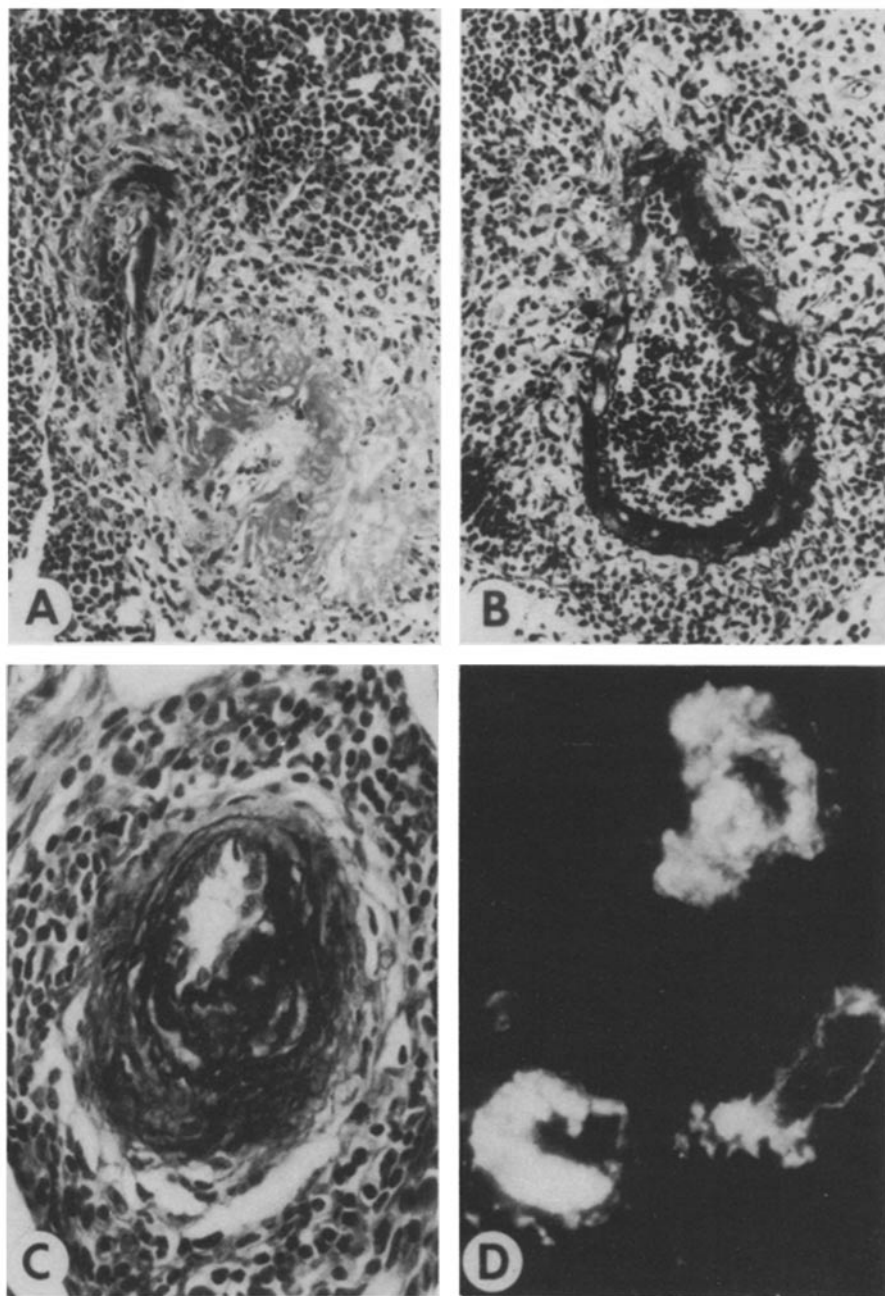


FIGURE 1 A-D. The spectrum of histopathology seen in NPA of SL/Ni mice. (A) Advanced NPA in the parametrium of a 12-mo-old SL/Ni female mouse (H & E). In the lower half of the photograph the media is completely replaced by fibrinoid material, which appears to be spreading from the media into the adventitia, while in the upper half there is necrosis of medial smooth muscle cells, infiltration of neutrophils, and abundant nuclear debris. (B) Advanced NPA of another older SL/Ni mouse (elastica and Masson trichrome). Note interruption of the elastic laminae and destruction of medial muscle cells evident in the left upper quarter of the photograph. (C) Another advanced vascular lesion found in the parametrial tissue (PAS stain) showing a late sclerosing stage of NPA. Multiplication of the elastic laminae is evident in this section. Encircling inflammatory cells are mainly composed of mononuclear cells. (D) Fluorescence micrograph of advanced vascular lesions in the parametrium of an old SL/Ni mouse showing dense granular depositions of mouse IgG in subintimal layer and in the media. See facing page for Fig. 1, E-H.

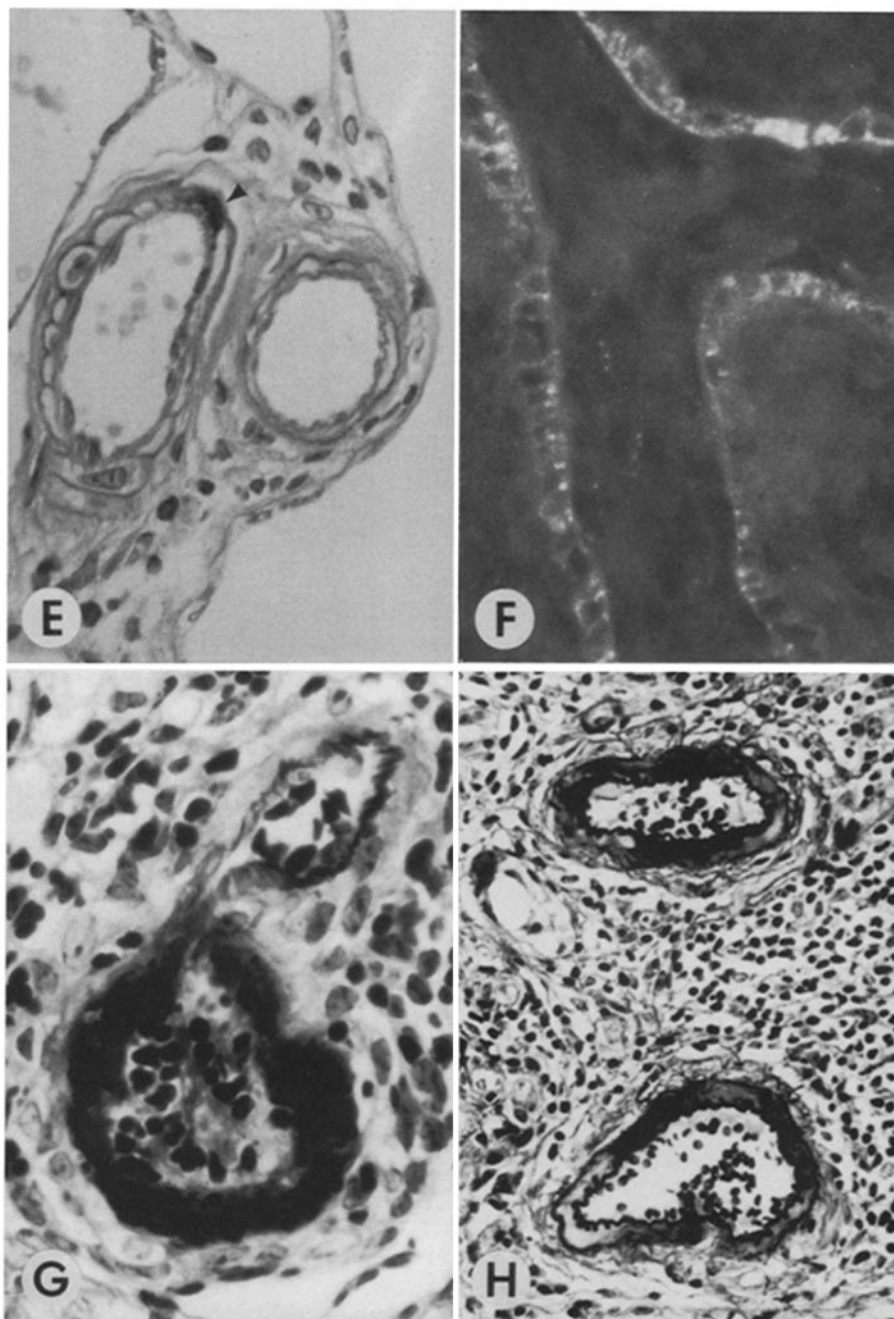


FIGURE 1 E-H. (E) An early stage of vascular change in a young SL/Ni mouse (PAS stain). Note thickening of the basement membranes around medial muscle cells and necrosis of an individual smooth muscle cell (*arrowhead*). There was no inflammatory cell infiltration. (F) IF micrograph of the parametrial arteries from a 1-mo-old SL/Ni female mouse showing fine granular deposition of IgG surrounding medial smooth muscle cells. (G) An early necrotic change of an arteriole found in the ovary (elastica and Masson trichrome). Note degeneration of the media and deposition of fibrinoid material in the subintimal layer seen in the center of the photograph. Edema and extravasation of red cells underneath the endothelium is also evident. (H) Two cut sections of the parametrial artery (elastica and Masson trichrome) showing necrosis of medial smooth muscle cells. Dying smooth muscle cells are stained dark and their nuclei are obscure.

NPA, dense granular depositions of IgG, IgM, and C3 were found within the PAS⁺ fibrinoid material in the thickened subintimal layer and in the media (Fig. 1D).

Histopathologic and Immunohistochemical Analysis of Earlier Lesions of NPA. The search for earlier lesions of NPA was done using young (1–3 mo old) SL/Ni female mice. Kidneys of these young mice showed no signs of glomerulonephritis or only the slightest proliferation of mesangial cells. The earliest detectable change of the vascular wall in affected SL/Ni mice was thickening of the basement membranes in the media with linear deposition of PAS⁺ material around smooth muscle cells and occasional necrosis of individual muscle cells without any leukocytic infiltration (Fig. 1E). IF studies of the uterine arteries in these young mice demonstrated linear or fine granular depositions of IgG and C3 surrounding smooth muscle cells in the media (Fig. 1F). In some instances, degeneration of medial smooth muscle cells was associated with extravasation of red cells and severe edema in the subintimal layer (Fig. 1G). However, there was no dense granular deposition of Igs in vascular walls in this early stage of NPA. The same type of early changes were also found in some older SL/Ni mice and abrupt necrosis of medial smooth muscle cells sometimes involved almost half of the cut area of an artery (Fig. 1H). However, even in such necrotic lesions inflammatory cells were not abundant.

Budding of C-type Particles from Arterial Smooth Muscle Cells and Binding of Host IgG and C3 on Their Surface. Tissue sections from the parametrium of SL/Ni mice at various ages were examined under an electron microscope. A large number of C-type MuLV particles were found budding from plasma membrane of arterial smooth muscle cells just before or at the onset of the initial change of arteritis (Fig. 2A). By immunoelectron microscopy, mouse IgG and C3 were found to be bound both to the area of the plasma membrane of the smooth muscle cells exhibiting budding virions as well as to the area remote from mature viral particles (Fig. 2B). In addition, degenerative changes of the antibody-bound smooth muscle cells were also found and were characterized by disintegrated plasma membrane, vacuolation underneath the cell membrane, disappearance of organelles, and depositions of electron dense material containing aggregated virions and mouse IgG either on the surface of or in the vicinity of the degenerating muscle cells (Fig. 2C).

Detection of Cytotoxic Anti-Ecotropic Virus Natural Antibodies in SL/Ni Sera. Even as early as 1 mo after birth, >50% of SL/Ni mice were producing large amounts of endogenous ecotropic MuLV in their spleen. By using XC plaque assays, we detected infectious ecotropic viruses in the spleen from 27 of 35 SL/Ni mice tested. Infectious xenotropic viruses were detected less frequently in older mice. Because arteritis affected primarily the mice in which the expression of high levels of ecotropic viruses was detected in the spleen (35), we chose the spleen for the source of endogenous ecotropic virus to establish virus-infected fibroblast lines. Since the ecotropic virus recovered from spleen cells of SL/Ni mice (SL/Ni ecotropic virus) was N-tropic, we cloned NIH 3T3 cells infected with the SL/Ni ecotropic virus and the most highly virus-producing clone, A-5, was used in further studies. A-5 cells expressed on their surface the MuLV envelope antigen which reacted exclusively with monoclonal antibody 24-8, but

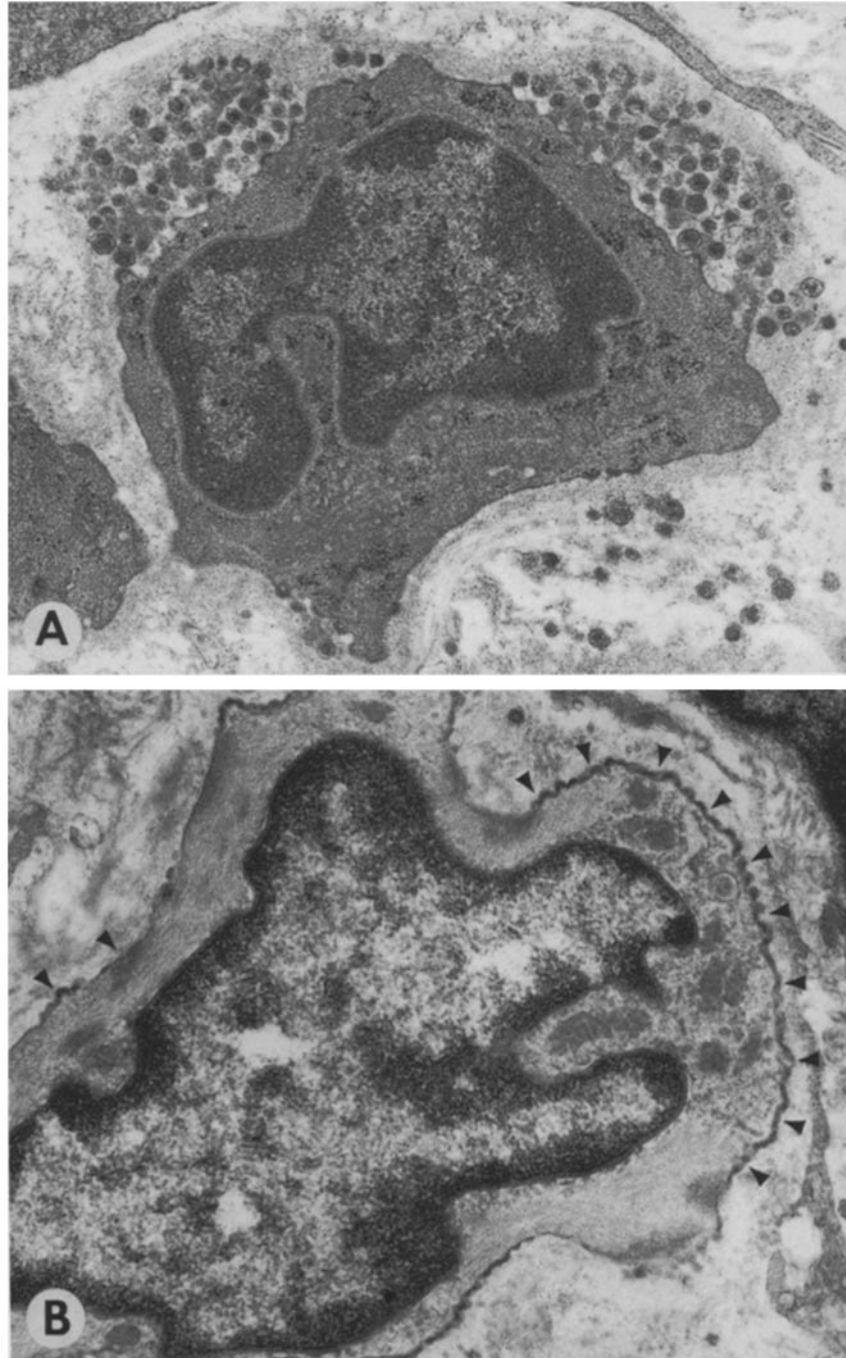


FIGURE 2 A and B. (A) Representative electron micrograph of parametrial arteries from young SL/Ni mice showing budding of numerous MuLV C-type particles from the plasma membrane of a smooth muscle cell ($\times 15,000$). Clusters of virions were found in the edematous, loosened matrix around the muscle cell. (B) Immunoelectron micrograph of a medial smooth muscle cell showing deposition of mouse IgG at the plasma membrane (*arrowheads*) ($\times 18,000$). See next page for part C.

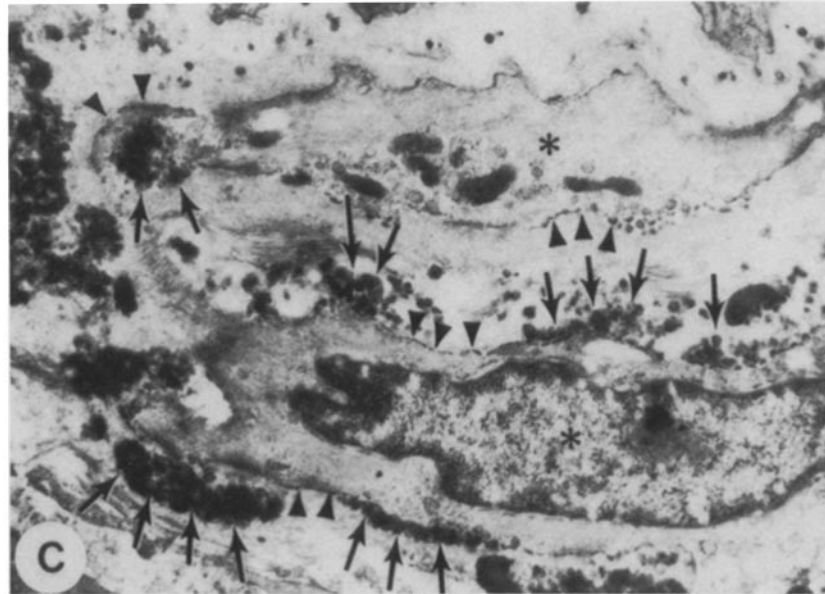


FIGURE 2 (C). Immunoelectron micrograph of the parametrial artery from a young SL/Ni mouse showing two degenerating smooth muscle cells (*) ($\times 12,000$). Note disintegration of the plasma membrane, vacuolation underneath the membrane, and severe edematous swelling of the surrounding matrix. Deposition of mouse IgG was still detectable on degenerating cell membrane (*arrowheads*), and there were dense aggregates of virions containing IgG in the vicinity of or on the surface of the muscle cells (*arrows*).

TABLE II
Reactivities of Anti-MuLV mAbs to A-5 Cells

Antibody	Reference	Virus specificity	IF on living A-5 cells
24-8	24	Endogenous eco* + MCF	+ [‡]
48	31	Exogenous Friend and Rauscher eco	- [§]
24-6	24	Xeno [†] + MCF	-
24-9	24	Xeno + MCF	-
18-6	24	Xeno + MCF	-
502	31	MCF	-
514	31	MCF	-
603	43	Xeno	-
613	43	Xeno	-
667	44	Wild mouse neurotropic eco	-
668	44	Wild mouse neurotropic eco	-
672	44	Wild mouse neurotropic eco	-
678	44	Wild mouse neurotropic eco	-

* Ecotropic MuLV.

[‡] Positive immunofluorescence.

[§] No detectable fluorescence.

[†] Xenotropic MuLV.

not with 24-9 or other monoclonal antibodies specific to xenotropic or MCF viruses (Table II). By fluorescent focus assays, culture supernatants of A-5 cells contained $\sim 10^4$ focus forming units per milliliter of infectious ecotropic virus,

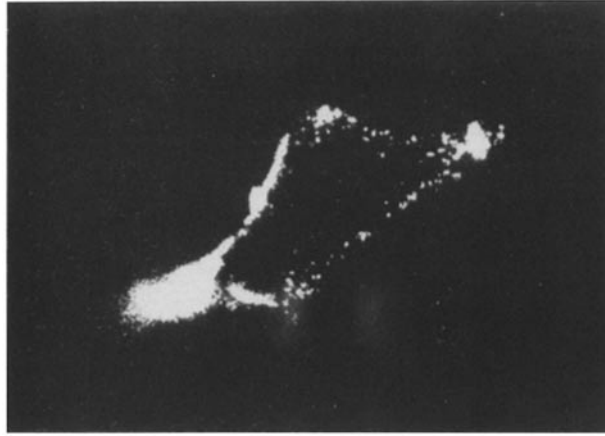


FIGURE 3. A fluorescence micrograph of a cell from the A-5 clone of NIH 3T3 cells infected with the endogenous ecotropic virus of SL/Ni mice. Live A-5 cells were incubated with SL/Ni serum and FITC-conjugated anti-mouse IgG antibody. Note fine granular cell surface fluorescence.

but no detectable xenotropic or MCF viruses.

Using this chronically infected fibroblast line we examined the possible presence of anti-ecotropic MuLV antibodies in SL/Ni sera. By IF staining of living A-5 and NIH 3T3 cells, naturally occurring IgG antibodies that bound specifically to the infected but not to the uninfected fibroblasts were detected in almost all SL/Ni mice tested (Fig. 3). These antibodies fixed murine complement and binding of mouse C3 was detected by IF. Anti-A-5 antibodies of IgM isotype were also found in some SL/Ni mice but their titer was lower than that of the IgG antibodies. No antibodies reacting with the A-5 cells were detectable in the control BALB/c sera.

Lysis of A-5 cells in vitro did take place when the cells were sequentially treated with SL/Ni sera and fresh BALB/c sera. Fig. 4 shows the results of microcytotoxicity assays. The sera from all 3 SL/Ni mice tested lysed A-5 cells in the presence of fresh BALB/c serum, while the serum from a control BALB/c mouse alone did not (Fig. 4A). A-5 cells were also lysed by SL/Ni sera in the presence of Guinea pig serum (Fig. 4B), whereas SL/Ni sera, as well as sera from BALB/c mice, did not lyse uninfected NIH 3T3 cells even in the presence of the Guinea pig serum (Fig. 4C).

Immunochemical Identification of gp70 as the Major Antigenic Molecule for the Natural Antibodies. In attempts to identify directly the molecules bearing the antigenic determinants for the natural antibodies, virions of the SL/Ni ecotropic virus were purified from culture supernatant of A-5 cells and SL/Ni sera were analyzed for reactivity to separated viral proteins by Western blot immunoassays. In a repeated series of assays, a mixture of pooled SL/Ni sera always showed strong immunoreactivity to gp70, while the control BALB/c sera did not react with any of the viral structural proteins.

For a detailed analysis of viral protein specificity of the anti-virus natural antibodies, a series of Western blot immunoassays was performed using individual sera from 14 female SL/Ni mice (9–14 mo old). Fig. 5 shows several representative patterns of the immunoreactivity to viral proteins exhibited by each SL/Ni serum. Almost all the SL/Ni mice tested had significant amounts of anti-gp70

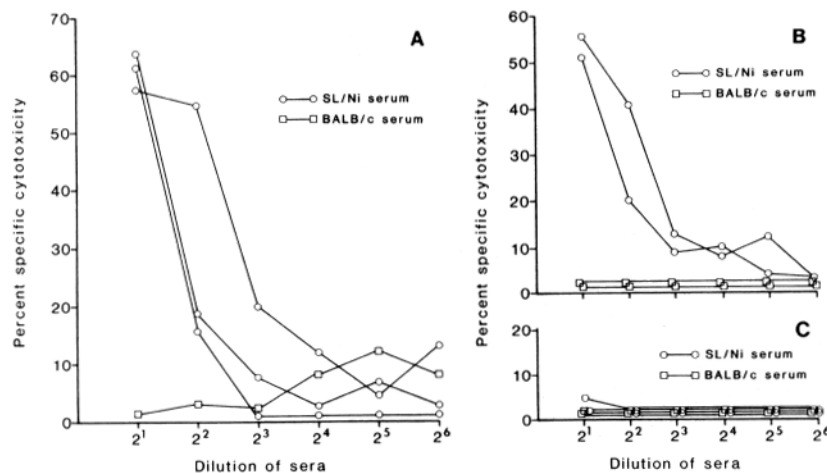


FIGURE 4. Microcytotoxicity assays showing titers of lytic antibodies in SL/Ni and BALB/c sera. (A) Lysis of A-5 cells by SL/Ni sera in the presence of fresh BALB/c serum as a complement source. BALB/c serum itself did not lyse A-5 cells. (B) Lysis of A-5 cells by SL/Ni sera in the presence of pooled Guinea pig serum as a complement source. (C) In this figure, target cells were uninfected NIH 3T3 instead of A-5. SL/Ni sera as well as BALB/c sera did not lyse the 3T3 cells even in the presence of pooled Guinea pig serum.

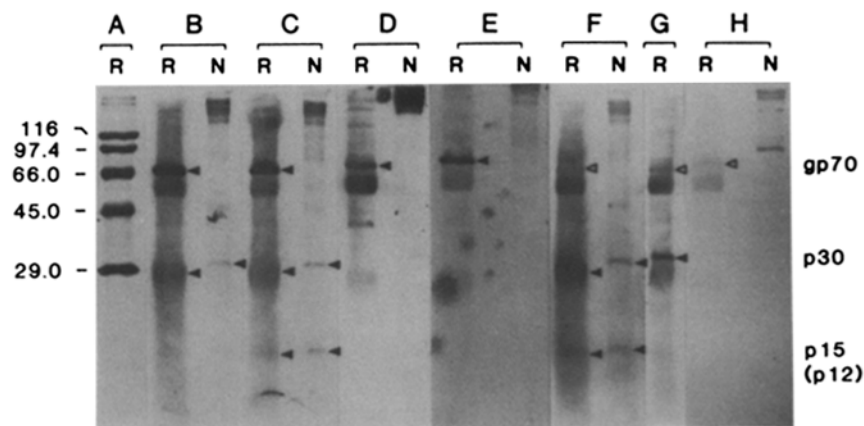


FIGURE 5. Western blot immunoassays using purified virions of the SL/Ni ecotropic virus. (A) Coomassie Brilliant Blue staining of molecular weight markers ($\times 10^{-3}$). (B-H) Immunodetection of blotted proteins with anti-mouse IgG. Purified virions were lysed with SDS sample buffer under a reducing (R) or a nonreducing (N) condition and were separated by SDS-PAGE. Slips of nitrocellulose paper on which separated proteins were blotted were incubated with 14 individual SL/Ni serum. Note strong reaction with viral gp70 in lanes B-E. Very weak or trace anti-gp70 reaction was seen in lanes F-H (open arrowheads). Anti-p30 reaction was more readily detectable with the unreduced lysate (N) of the virus (B, C, F, and G). Broad and relatively faint reaction detectable beneath the band of gp70 might represent the band of degraded or truncated envelope glycoprotein because the same band was detectable with the anti-Rauscher gp70 antiserum and anti-gp85 mAb 24-8, but not with anti-p30 antibody R187. However, the true nature of this fraction was unclear.

antibodies, although the intensity of the bands differed from one mouse to another (Fig. 5, B-E). On the other hand, 3 of the 14 mice showed very weak or trace anti-gp70 reaction (Fig. 5, F-H). In addition, some SL/Ni mice possessed

TABLE III
Influence of Viral Protein Specificity of Anti-MuLV Natural Antibodies on the Development of NPA in SL/Ni Mice

Animal number	Viral protein specificity of natural antibodies*			Lym- phoma [‡]	NPA [‡]	
	gp70	p30	p15(p12)		Paramet- rium and/or ovary	Other organs
CF3014	— [§]	—	—	+	—	—
CF3028	+	—	—	+	+	+ (1) [†]
CF3067	++	+	+	—	—	+ (2)
CF4011	++	+	—	—	+	+ (2)
CF4046	++	—	—	+	—	—
CF4104	++	+	—	—	+	+ (1)
CF4112	— [§]	++	+	—	—	—
CF4128	+	—	—	+	+	+ (2)
CF4129	+	—	+	—	+	+ (1)
CF5128	++	+	+	—	+	+ (2)
CF5142	— [§]	+	+	—	—	—
CF5235	++	—	—	—	—	—
CF6015	++	—	+	—	+	+ (2)
CF7021	++	—	+	—	+	—

All SL/Ni mice in this table were affected by glomerulonephritis.

* See Materials and Methods for the grading of immunoreactivity.

[‡] In the columns of lymphoma and NPA: +, affected; —, unaffected.

[§] Very weak or trace reactivity to gp70 in Western blotting.

[†] Number of organs affected by NPA other than the parametrium or ovary.

anti-core protein antibodies. Among them, anti-p30 was detected in 6 of the 14 SL/Ni mice (Fig. 5, B, C, F, and G). Furthermore, reactivity to a polypeptide having a mol wt <30,000 was found in 7 SL/Ni mice (Fig. 5, C and G). The molecular weight of the polypeptide (10,000–15,000), the presence of the fraction in both the reduced and unreduced lysates of the virions, and lack of the reactivity of monoclonal anti-p15E antibody 372 to this fraction, all suggested that this low molecular weight protein was p12 or p15, which are encoded by the viral *gag* gene (36).

Table III summarizes the results of the immunochemical analysis with histopathologic findings of the 14 SL/Ni mice tested. All the mice were affected by severe membranoproliferative glomerulonephritis and four were affected by lymphoma involving primarily the spleen and lymph nodes. Arterial lesions of various stages were found in 9 of the 14 mice, and their distribution by organ was quite similar to that summarized in Table I. As shown in Table III, 11 of the 14 SL/Ni mice had significant level of anti-gp70 antibodies and all the 9 mice affected by arteritis were anti-gp70⁺.

Demonstration of Ecotropic Viral Envelope Antigen in Arterial Walls of SL/Ni Mice. Two type-specific mAbs, 24-8 and 24-9, of the panel of anti-MuLV antibodies in Table II were used for IF study of arterial walls of SL/Ni mice. Since both the monoclonals were murine IgG, and most of the gp70 expressed in arterial walls of SL/Ni mice was expected to have made complexes with mouse

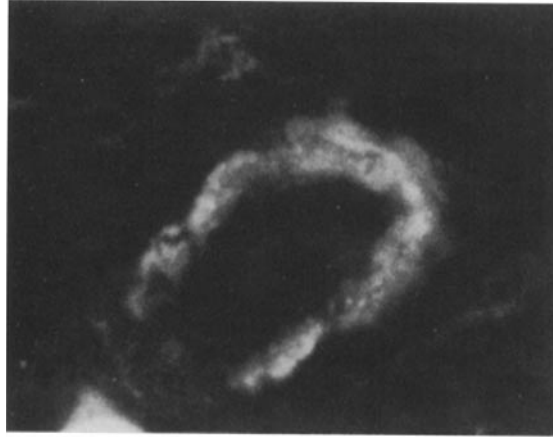


FIGURE 6. A fluorescence micrograph showing depositions of the MuLV envelope antigen in arterial wall of a SL/Ni mouse that reacted with mAb 24-8.

IgG, we biotinylated the purified antibodies and an indirect IF technique using FITC-conjugated avidin D was used.

By using mAb 24-8, viral envelope gp85, a complex of gp70 and p15E, was demonstrated in arterial walls of SL/Ni mice. The viral envelope antigen was detected either in a coarse granular pattern along with PAS⁺ fibrinoid material in the media (Fig. 6) or in a fine granular pattern around smooth muscle cells in the media. On the other hand, no reactivity of mAb 24-9 with frozen sections from the same arteries was detected, confirming the lack of xenotropic viral gp70 in these lesions.

Discussion

In the present study we examined the histologic, immunohistochemical, immunoelectron microscopic, and serologic parameters expressed by SL/Ni strain of mice, which spontaneously develops necrotizing polyarteritis involving various organs (Table I). In the examination of early lesions in young SL/Ni mice, C-type MuLV particles were found budding from plasma membrane of arterial smooth muscle cells just before or at the onset of vascular injury (Fig. 2A) and depositions of mouse IgG and C3 were demonstrated on the surface of the muscle cells (Fig. 2B). The antibody-bound smooth muscle cells lost the integrity of their plasma membrane and showed vacuolation underneath the membrane and disappearance of cellular organelles (Fig. 2C). Degeneration and necrosis of smooth muscle cells in the media were the earliest detectable histopathologic changes due to arteritis and they seemed to occur without dense infiltration of inflammatory cells (Fig. 1, E, G, and H). Although there is no direct evidence showing the presence of membrane-attack complex (C₅₆₇₈₉) of murine complement on plasma membrane of the damaged smooth muscle cells, these *in vivo* findings strongly suggested that antibody-dependent, complement-mediated membrane damage of the smooth muscle cells leading to degeneration and necrosis of these cells was the main pathogenetic mechanism producing NPA in SL/Ni mice.

To induce destruction of the vascular component, SL/Ni mice must have cytotoxic antibodies that react with some membrane-bound antigens expressed

on the smooth muscle cells. We demonstrated the presence of natural antibodies in SL/Ni sera that bound specifically to endogenous ecotropic MuLV of these mice. Sera from SL/Ni mice could lyse the infected fibroblast in the presence of murine complement, and therefore, at least some of the antiviral natural antibodies were cytotoxic. Sissons et al. (37, 38) demonstrated that lysis of measles virus-infected HeLa cells with human antibody and fresh human serum was mediated by alternative complement pathway. Our preliminary experiments suggest that the fixation of murine C3 on antibody-bound A-5 cells requires the presence of Ca^{2+} and, therefore, the lysis is probably mediated by classical complement pathway. Of course, there must be a sufficient amount of complement in the serum, in addition to the presence of antibodies, to induce the cytolysis. In fact, SL/Ni mice expressed sufficient levels of complement since the amount of C3 in younger (<9 months in age) SL/Ni mice was 50–110% of that of control BALB/c mice (39).

The results of Western blot immunoassays (Table III) clearly showed that the major molecule bearing the antigenic determinants for antiviral natural antibodies in SL/Ni mice is viral envelope gp70, and that there was no correlation between the presence of anti-p30 or anti-p15 (anti-p12) antibodies in the serum and the development of arteritis. Statistically, the correlation between the presence of anti-gp70 antibodies and the development of arteritis was weakly significant ($p = 0.028$ by Fisher's method), and two mice were not affected by arteritis even though they showed strong anti-gp70 reaction. However, we cannot deny the essential role of anti-gp70 antibodies because the development of arteritis may also depend on the expression of ecotropic viral gp70 on the surface of arterial smooth muscle cells and the presence of antiviral antibodies may be a necessary prerequisite that has been met in almost all SL/Ni mice before the onset of arteritis. It is therefore very important to emphasize the result that the three SL/Ni mice having no or a very low level of anti-gp70 antibodies did not develop arteritis in spite of the presence of anti-core antibodies in their serum.

Since the correlation between the presence of anti-gp70 antibodies and the development of NPA was demonstrated, it became very important for the proof of the above-described pathogenetic mechanism to test the presence of viral envelope gp70 in arterial walls of SL/Ni mice. By IF using monospecific antisera to Rauscher MuLV structural proteins, Yoshiki et al. (14) had demonstrated gp70, p30, and p15E deposited in affected arteries of SL/Ni mice. However, a more specific detection of ecotropic viral envelope antigen was required because there was a possibility that the virions budding from arterial smooth muscle cells were antigenically quite different from, and therefore not crossreactive with, the ecotropic virus isolated from the spleen. Using type-specific mAbs we detected the deposition of ecotropic viral, but not xenotropic viral, envelope antigen in arterial walls of SL/Ni mice. These results thus indicate that the arteritis in SL/Ni mice requires both the expression of endogenous ecotropic virus in vascular smooth muscle cells as well as the presence of free antibodies reacting with the viral gp70.

So far the deposition of circulating ICs in vascular walls has been the most frequently postulated mechanism for the production of necrotizing arteritis (3–6). Since the circulating ICs in most cases had no immunologic relationship to

any vascular components, the pathogenicity of ICs was thought to be determined chiefly by the amount and size of the complexes, and by the type of Igs involved (6). For example, in experimental models of serum sickness (4, 5), prolonged daily exposure of the animals to low levels of ICs only caused glomerulonephritis, whereas one-shot exposure with large amounts of ICs induced arteritis as well as nephritis. The same factors determining the development of vasculitis were indicated by Berden et al. (40) in spontaneous murine models of SLE. In SL/Ni strain, circulating ICs have been detected in older animals (12, 39), and almost all the SL/Ni mice over the age of 10 mo are affected by severe glomerulonephritis due to the deposition of ICs (10–14). However, the amount of circulating ICs in SL/Ni female mice that were younger than 4 mo was within the level of control BALB/c mice (39), and no signs of glomerulonephritis were found in these younger SL/Ni mice (11, 12). Nevertheless, the earliest changes of arteritis, including necrosis of medial smooth muscle cells, are occurring in this very early life of SL/Ni mice. Moreover, IF studies of the early arterial lesions revealed linear depositions of IgG and C3 on the surface of medial smooth muscle cells instead of the characteristic dense depositions of ICs (Fig. 1*F*). Therefore, the early necrotic lesions of NPA in SL/Ni mice appear to be produced primarily by the lytic mechanism rather than by IC-mediated mechanisms. Of course, it is possible that circulating ICs play a role in production of the advanced lesions of NPA because the advanced lesions are found in older animals and older SL/Ni mice do possess ICs in their serum. In fact, vascular lesions of advanced stages represented by Fig. 1, A–C, are quite similar to those of the experimental acute serum sickness, both of which are characterized by dense infiltration of inflammatory cells, deposition of fibrinoid material, and destruction of elastic laminae, and IF studies of the advanced lesions have clearly shown the depositions of ICs in affected vascular walls (Fig. 1*D*). However, even in older SL/Ni mice neither the amount of ICs nor the molecular size of the complexes correlated with the development of NPA, although the amounts of ICs did correlate with the development and severity of glomerulonephritis (39). Moreover, all the SL/Ni mice in Table III clearly have circulating ICs since they are all affected by membranous or proliferative glomerulonephritis. Nevertheless, three of them that have no detectable anti-gp70 antibodies are not affected by arteritis. This evidence suggests that the free, cytotoxic antibodies are also essential, as are circulating ICs, for production of the advanced NPA. Thus, antibody-dependent, complement-mediated cytotoxic reactions in the media may liberate several chemical mediators of inflammation and consequent increase in vascular permeability may induce the deposition of circulating ICs in arterial walls. Extravasation of red cells and marked subendothelial edema shown in Fig. 1*G* may support such a speculation. Therefore, circulating ICs may act as accelerators of vascular injury rather than as initiators in SL/Ni mice (35).

In contrast to other spontaneous murine models of SLE (8), late onset of glomerulonephritis and resultant longer mean survival time are remarkable features of SL/Ni mice. Although small amounts of IgG and IgM deposits were detectable in the mesangial portion of the glomeruli in <50% of the SL/Ni mice examined at 4 mo of age (11), fully developed membranous and/or proliferative glomerulonephritis with dense granular depositions of Igs was only found after

6–7 mo of age. This is clearly related to late (after 5–6 mo in age) increase in the amount of circulating ICs (39). On the other hand, early onset of glomerulonephritis associated with high levels of circulating ICs is one of the common characteristics of MRL/1, BXSB/Mp male, and F1 hybrid of New Zealand Black and New Zealand White mice (7, 8). Nevertheless, the development of NPA is uncommon among these strains, except in MRL/1, in which a high frequency (~75%) of NPA was observed in older animals (8). The development of NPA in older MRL/1 mice is associated with the >10-fold elevation of the amount of circulating ICs that occurs between 2 and 4 mo of age (40). Such sudden elevation of circulating IC levels has never been observed in SL/Ni mice (39). Thus, this evidence again supports the hypothesis that the lytic reaction in the vascular media may induce the deposition of circulating ICs into the specific site of the vasculature, even if the amount of ICs is so small that the complexes themselves cannot induce arteritis.

Finally, the gp70 antigens expressed in sera of all lupus mice except SL/Ni are antigenically closely related to that of the infectious xenotropic viruses of New Zealand Black mice, and these mice produce anti-gp70 antibodies reactive with the same specific gp70 (41). In contrast, the MuLV expressed in the vascular media of SL/Ni mice were found to be ecotropic, and ecotropic viral envelope antigens were detected along the basement membrane and mesangial site of the lupus-like glomerular lesions in SL/Ni mice (Miyazawa, M., unpublished observation). Therefore, the type of endogenous MuLV involved in the production of autoimmune lesions in SL/Ni mice is completely different from that of other lupus mice.

In conclusion, the SL/Ni strain of mice is a unique animal model of autoimmune necrotizing arteritis. In this strain, the expression of endogenous ecotropic virus and antibody-dependent, complement-mediated destruction of arterial smooth muscle cells may play crucial roles in producing the vascular lesions. This system may be useful in studying the pathogenetic mechanisms involved in human vasculitides of unknown etiology. One such acute arteritis of infants, Kawasaki disease, involving coronary arteries and sometimes causing fatal aneurysms, has recently been associated with retroviral infection (42).

Summary

The SL/Ni strain of mice spontaneously develops a necrotizing polyarteritis (NPA) that is histologically quite similar to human polyarteritis nodosa. This NPA most frequently affected parametrial tissues and/or ovaries of females and small arterioles of the major salivary glands. Electron microscopic studies of early arterial lesions revealed massive budding of C-type particles from arterial smooth muscle cells just before or at the onset of arteritis. In addition, binding of mouse IgG and C3 to the plasma membrane of virus-producing smooth muscle cells was shown by immunoelectron microscopy. Antibody-bound muscle cells showed disintegration of their plasma membrane, but degeneration and necrosis of muscle cells were not associated with dense infiltration of neutrophils.

SL/Ni mice had natural antibodies that bound specifically to a fibroblast cell line infected with an endogenous ecotropic murine leukemia virus (MuLV) recovered from a SL/Ni mouse. Most of the natural antibodies were cytotoxic

in the presence of murine complement. Western blot immunoassays revealed that among 14 SL/Ni female mice tested, all of the 9 mice that were affected by arteritis had anti-gp70 antibodies, while the 3 anti-gp70⁻ mice were not affected. The presence of anti-p30 or anti-p15 (anti-p12) antibodies, which were also detected in some SL/Ni mice, did not correlate with the development of arteritis.

These results strongly support the hypothesis that NPA in SL/Ni mice is mediated by the lysis of arterial smooth muscle cells due to the deposition of cytotoxic natural antibodies directed to cell membrane-bound gp70 molecules of an endogenous ecotropic MuLV.

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