The Role of Vascular Smooth Muscle Cells in Experimental Autoimmune Vasculitis

I. The Initiation of Delayed Type Hypersensitivity Angiitis

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The destruction of vascular smooth muscle cells (VSMCs) in autoimmune arteritis is a poorly understood phenomenon. For evaluation of the cellular interactions that may contribute to vasculitis, the immunobiology of VSMCs and lymphocytes was explored *in vitro*. Primary VSMC cultures were established, and the interaction of these cells (from normal or autoimmune mice) with lymphocytes was then assessed. Specifically, splenocytes from MRL/lpr or C_3H mice were cocultivated with MRL/lpr or C_3H VSMCs. Massive mononuclear cell clusters from normal and autoimmune mice enveloped MRL/lpr VSMCs, which culminated in the detachment of MRL/lpr VSMCs from the culture plate. In contrast, the interaction of SPs from either normal or autoimmune mice did not encompass or destroy normal VSMCs. Further investigation indicated that MRL/lpr, but not C_3H, VSMCs spontaneously expressed Ia and released Il-1 like factor(s), which may be at least two mechanisms by which MRL/lpr VSMCs stimulate the *in vitro* mononuclear cell influx. As a result of these studies, a novel mechanism for the induction of mononuclear cell autoimmune vasculitis is proposed. VSMCs derived from autoimmune mice may stimulate a mononuclear inflammatory cell phlogistic response which culminates in VSMC autodestruction. (Am J Pathol 1984, 117:380–390)

THE PATHOGENIC MECHANISMS that induce vasculitis are still inadequately defined, despite the fact that this lesion has been recognized since 1866.[1] Perhaps the best-studied mechanism is the neutrophilic, immune-complex-mediated vasculitis involved in Type III hypersensitivity, as described by Dixon in 1963.[2] The sequence of events can be summarized as follows: Antigen interacts with basophils or mast cells which have IgE on their surfaces. These cells release platelet-activating factor (PAF), which causes platelets to release vasoactive amines. These amines cause endothelial cell separation and allow greater than 19 S immune complexes to be deposited on the luminal aspect of the vessel wall. As a result, the classic complement pathway is stimulated, and chemotactic factors are released. Neutrophils are attracted to the site, which release lysosomal enzymes that destroy the vascular wall, causing fibrinoid necrosis.

There is a second group of vasculitides unexplained by this mechanism alone. These lesions are characterized morphologically by lymphocyte and macrophage perivascular cuffs, which initiate vascular wall destruction by first infiltrating the vascular adventitia and, secondly, the media. Very little, if any, neutrophilic participation is observed. This type of vasculitis, lymphocyte-predominant or mononuclear-cell vasculitis, has been observed principally in cutaneous vasculitides[3,4] and in selected autoimmune (AI) disease[5,6] in the human.

The pathophysiologic mechanisms that underlie mononucler cell vasculitis are poorly understood because precisely which cells are involved has not been

Supported by Grant 2-S07-RR07047-17 from the National Institutes of Health (C.F.M.), Grant IM-300A from the American Cancer Society (C.L.R.), and Grant 2R01-CA32155 from the National Institutes of Health (C.L.R.).

Accepted for publication June 21, 1984.

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defined. To date, evidence suggesting that cell-mediated immunologic mechanisms induce vasculitis has been derived from histologic, immunocytochemical,[6] and *in vivo* studies.[7] Our approach was to design *in vitro* methods to dissect the cellular mechanisms which might mimic the lesion observed morphologically. We chose the MRL/Mp-lpr/lpr (MRL/lpr) mouse, which expresses clinical, serologic, and histologic features of systemic lupus erythematosus and rheumatoid arthritis in the human.[8,9] These animals develop immune-complex-mediated neutrophilic vasculitis at 16 weeks of age.[10] Recent studies have focused on the relationship of serologic abnormalities to immune-complex-mediated vasculitis development.[10] Rather than investigating the maturation of vasculitis, we studied how angiodestruction was initiated. Chronologic evaluation of vascular tissue from 8–16-week-old MRL/lpr mice indicated that these mice developed perivascular mononuclear inflammatory cell cuffing, which progressed to medial angiomyodestruction, as early as 12 weeks of age. These histologic features were similar to those in the vascular disease observed in the human.[5,6] Because of these unique morphologic features, we chose to address the question of whether or not vascular smooth muscle cells (VSMCs) had an active/passive role in the initiation of the observed *in vivo* mononuclear inflammatory cell influx. We developed an *in vitro* VSMC model system with which to evaluate lymphocyte/VSMC interactions.

Our studies suggest that VSMCs from MRL/lpr mice with autoimmune disease may initiate an autologous splenocyte (SP) response culminating in MRL/lpr VSMC autodestruction. Further investigation into the potential mechanisms by which MRL/lpr VSMCs stimulate the lymphocyte and macrophage influx indicate that MRL/lpr VSMCs spontaneously release an interleukin-1-like factor and express Ia.

Materials and Methods

Mice

Twenty-week-old C_3H/Hej (C_3H) or MRL/Mp-lpr/lpr (MRL/lpr) female mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. The C_3H/Hej inbred strain and the MRL/lpr congenic strain are syngeneic at the H-2k histocompatibility locus.

VSMC Cultures

VSMC tissue cultures were grown according to methods described by Gimbrone[11] and Chamley-Campbell.[12] Tissue culture medium and supplements were purchased from GIBCC Laboratories (Grand Island, NY). Mesenteric and aortic vessels were removed aseptically from C_3H or MRL/lpr mice. Adventitial fat and connective tissue were stripped from vessels using a dissecting microscope and fine watchmaker forceps. Vascular smooth muscle cells were enzymatically dispersed from the exterior intact vessel wall with the use of 0.05% collagenase (Sigma Chemical Company, St. Louis, Mo) and 0.025% elastase (Sigma Chemical Company). Primary cells were cultured in 6-well (35-mm) plates (Costar, Cambridge, Mass) with complete medium consisting of Dulbecco's modified Eagle's medium, 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin–100 μg streptomycin, and 2 mM L-glutamine. Cells were subcultured by incubation at 37 C with 0.05% collagenase and 0.025% elastase for 10 minutes and replated on 24-well (15-mm) Linbro plates (Flow Laboratories, McLean, Va). Thermanox 13-mm plastic coverslips (Flow Laboratories) or 13-mm glass coverslips (Bellco Glass, Vineland, NJ) were placed in duplicate wells. The VSMCs grown on plastic coverslips were subsequently used for the scanning electron microscopic studies. The VSMCs cultured on glass coverslips were used for the fluorescence microscopic studies. Both studies are described below.

Angiotensin Assay

Two groups of VSMCs were examined: 1) primary cells, 5 days after enzymatic dispersement, and 2) first through sixth passage culture cells, 3 days after subculture. Both groups were incubated with 1×10^{-6} M Asp,[1] Ile[5] angiotensin II (ATII) (Sigma Chemical Company) for 0–10 minutes.[13] Contraction by VSMCs was evaluated by phase microscopy.

Splenic Leukocyte Isolations

Spleens were aseptically removed from C_3H or MRL/lpr mice. Splenocytes (SPs) were teased with watchmaker forceps into Petri dishes containing RPMI. The cell suspensions were subsequently treated with 0.83% ammonium chloride for 30 seconds for removal of red blood cells, washed several times with phosphate-buffered saline (PBS), and diluted to a 1×10^6 cells/ml cell suspension.

Scanning Electron Microscopy

First through third passage MRL/lpr and C_3H VSMCs were grown on Thermanox 13-mm coverslips in 24-well Linbro plates. One million splenocytes derived from either C_3H or MRL/lpr mice were cocultivated with either C_3H or MRL/lpr VSMCs. The

SP/VSMC interactions were observed by light and phase microscopy at 2-hour intervals up to 18 hours. Coverslips containing VSMCs and SPs from each 2-hour time interval between 2 and 18 hours were fixed in cold 25% Karnovsky's fixative[14] for 10 minutes and washed with 0.1 M cacodylate buffer (pH 7.4–7.6) at 24 C. Samples were progressively dehydrated with alcohols, critical-point-dried, and sputter-coated with gold–palladium. An ISI DS 130 scanning electron microscope was used for examination of the specimens.

Each experiment included the four possible SP/VSMC cocultivation populations. Specifically, MRL/lpr VSMCs cocultivated with either MRL/lpr SPs or C_3H SPs and C_3H VSMCs cocultivated with either MRL/lpr or C_3H SPs were examined. The MRl/lpr VSMC cocultivation experiments were repeated four times in duplicate. The C_3H VSMC cocultivation experiments were repeated twice, in duplicate.

We examined each 2-hour sample from all experiments: 1) to assess SP/SP and SP/VSMC intercellular interactions, and 2) to quantify the number of SPs adhered to VSMCs. The number of SPs adhering to 30 randomly selected VSMCs was counted from each 2-hour coverslip. The mean and standard deviation (SD) for each time period were derived. Since the SP/VSMC interaction ceased by 16 hours, the mean SPs/VSMC for each 2-hour time period bewteen 2 and 14 hours was analyzed by the analysis of variance (ANOVA) statistical test.

Mitogen

Concanavalin-A (Con-A) was used at a final concentration of 2 μg/ml (Calbiochem, La Jolla, Calif).

Thymocytes

Thymuses from 4–6-week-old Balb/c mice were aseptically removed. Thymocytes were teased with watchmaker forceps into Petri dishes containing PBS. The cell suspensions were washed several times with PBS and diluted to 1.5×10^6/well.

Interleukin-1 (IL-1) Assay

One and a half million thymocytes/well were incubated +/− Con-A +/− supernatants from 2-day MRl/lpr or C_3H VSMC cultures. The thymocytes were cultured for 48 hours in 96-well flat-bottom microtiter plates, pulsed with 1 μCi ^{3}H-thymidine (New England Nuclear, Boston, Mass) for 15–18 hours and harvested using a MASH apparatus (M A Bioproducts). The filters were dried, placed into 3-ml Aquasol scintillation fluid (New England Nuclear), and the incorporation of radioactivity was assessed with a Beckman LS 6800 scintillation counter (Beckman Instruments, Wakefield, Mass). The assay was repeated three times in triplicate.

Antibodies

Murine monoclonal antibody to Ia^k was concentrated from culture supernatants of hybridoma cell line 10-2-16. Murine monoclonal antibody to IgG2A was purchased from Becton-Dickinson and used as a negative control. Rabbit anti-mouse F $(ab')_2$ conjugated to fluorescein isothiocyanate (FITC) (Cappell Laboratories, Cochranville, Pa) or Protein-A FITC (Pharmacia Chemical, Piscataway, NJ) were used as second antibodies for determination of the binding of the primary anti-Ia^k monoclonal antibody. FITC-conjugated rabbit anti-mouse F $(ab')_2$ antiserum was used at a final dilution of 1:30. Protein-A FITC was used at a final dilution of 1:40.

Analysis of Ia Expression by VSMCs

Two indirect immunofluorescence techniques were used for analysis of the expression of Ia by MRL/lpr and C_3H VSMCs. These VSMCs were first evaluated by fluorescence microscopy and secondly by flow cytometry.

Evaluation of Ia Expression by Fluorescence Microscopy

First through third passage MRL/lpr or C_3H VSMCs were cultured on 13-mm glass coverslips. The coverslips were fixed in −20 C acetone for 10 minutes and rinsed with cold PBS. Application of either monoclonal anti-Ia^k or monoclonal anti-IgG2A antibody were added to the coverslips for 30 minute at room temperature (24 C). Each coverslip was washed three times, for 5 minutes per wash, with PBS. A second antibody, FITC-conjugated rabbit anti-mouse F $(ab')_2$ or Protein-A FITC, was applied to each slide at room temperature for 30 minutes. After three 5-minute washes with PBS, each slide was air-dried, and a coverslip with Permount was placed over each sample. The samples were examined with an inverted Zeiss IM 35 light, phase, and epifluorescent microscope.

Evaluation of Ia Expression by Flow Cytometry

VSMCs were cultured on 40-μ microspheres as described.[15] Briefly, 1 g of sterile 40-μ polystyrene beads

(Duke Scientific, Palo Alto, Calif) was washed four times in sterile PBS (pH 7.4) for removal of the sodium azide. The beads were diluted with PBS to a final concentration of 2 mg/ml. One milliliter (2 mg/ml) of 40-μ polystyrene microspheres and 1 ml fibronectin (50 μg/ml) (Sigma) were incubated at 37 C for 30 minutes in a 15-ml plastic centrifuge tube. One milliliter of 100% FCS was added to the microsphere/fibronectin combination, and the resulting FCS/microsphere/fibronectin mixture was reincubated for 30 minutes at 37 C. Next, this mixture was centrifuged at 800 rpm for 5 minutes, and the supernatant was decanted. The FCS/microsphere/fibronectin combination was resuspended in 1 ml of complete medium, and 0.25 ml was aliquoted into #1007, 60 × 15-mm Petri dishes (Falcon, Oxnard, Calif). Two hundred thousand VSMCs were added to each 60 × 15-mm Petri dish. Two-thirds of the medium was changed every 2–3 days. Two days prior to flow-cytometric analysis, the Petri dishes containing the microsphere/VSMC mixture were placed on an orbital Micro-Shaker II (Dynatech Laboratories, Alexandria, Va) at 51 rpm.

Preparation of VSMC/microspheres for flow cytometric analyses was as follows: The VSMC/microspheres were removed from the culture plate surface with a rubber policeman, followed by gentle trituration with a Pasteur pipette. The VSMC/microspheres were rinsed three times in sterile PBS and labeled with the use of the indirect immunofluorescence technique for single-cell suspensions as previously described.[16] The same immunologic reagents were utilized as described for evaluation of Ia expression by fluorescence microscopy. Samples were analyzed in an Ortho Spectrum III (Ortho Diagnostic Systems, Inc., Westwood, Mass) with a 200-μ orifice to permit passage of 40-μ microspheres with VSMCs through the flow cytometer without clogging and disrupting the analysis. The laser was adjusted to provide 30 mm of power at 488 nm. Data were collected on the cell volume and the immunofluorescence of each cell passing through the orifice. VSMC/microspheres formed a distinct cluster on the plot of volume versus fluorescence intensity.[15] The samples were gated using the light scatter detected along the laser beam (forward light scatter). The green fluorescence of gated VSMC/microspheres was measured 90 degrees to the laser beam (right angle scatter). Gates were set to exclude VSMCs alone, debris, and/or microspheres alone from analysis. These particles had less right-angle light scatter than microspheres with adherent VSMCs. Data resulting from the cell number versus fluorescence plot were displayed as a histogram. Fluorescence intensity was exhibited on the x axis, and the number of cells exhibiting fluorescence, on the y axis. Anti-Ia fluorescence was always compared with the nonspecific background fluorescence of fibronectin/FCS/microspheres alone and with the nonspecific binding of IgG2A.

Results

Evaluation of VSMC Morphology and Function

VSMCs were enzymatically dispersed from mesenteric and aortic vessels derived from either MRL/lpr or C_3H mice. Brief collagenase/elastase treatment yielded an homogenous VSMC population; however, prolonged treatment produced a mixed population of endothelial cells and VSMCs. Endothelial cells were distinguishable by their polygonal, platelike shape, 30 × 50-μ size, and raised centrally located oval nuclei. They formed small clusters in culture.[11]

VSMCs were spindle- or ribbon-shaped cells, much larger than endothelial cells (100–200 μ × 10–15 μ), with oval or sausage-shaped nuclei containing two or more nucleoli. These cells appeared in culture after 4–5 days and began to dedifferentiate at 9–12 days. In their dedifferentiated, synthetic, and replicative stage,[12] they became very large (up to 400 μ) and contained a single nucleolus. Under phase-contrast microscopy, their perinuclear granular appearance was visible, as were their prominent longitudinal myofilaments. The cells grew in clusters and assembled in a crisscross pattern during the subconfluent growth phase. Monolayer confluency of VSMCs could require up to 3 weeks.

Selective treatment of primary VSMC cultures with enzymes, such as brief incubation with 0.06% trypsin for 1–2 minutes after 6–8 days, removed contaminating endothelial cells.[13] VSMCs were subcultured with 0.05% collagenase/0.025% elastase.

To confirm VSMC functional integrity *in vitro*, primary and subcultured cells were evaluated on the basis of their contractile response to angiotensin II administration.[13] Primary cells, 5 days after dispersion, were assessed. Seventy-five percent of the differentiated VSMCs contracted to approximately 60% of their former size after 6 minutes of ATII administration. Characteristic cellular evaginations were present (Figure 1).

Subcultured cells, three to five days post subculture, responded with the same intensity and time course as the primary cells to ATII administration. These findings concurred with Brock et al, who determined that, *in vitro*, VSMC contractile response to ATII administration remained intact up to the 32nd passage.[17]

These studies showed that the VSMCs were morphologically homogenous and functionally intact. We then analyzed the interaction of VSMCs with SPs *in vitro*.

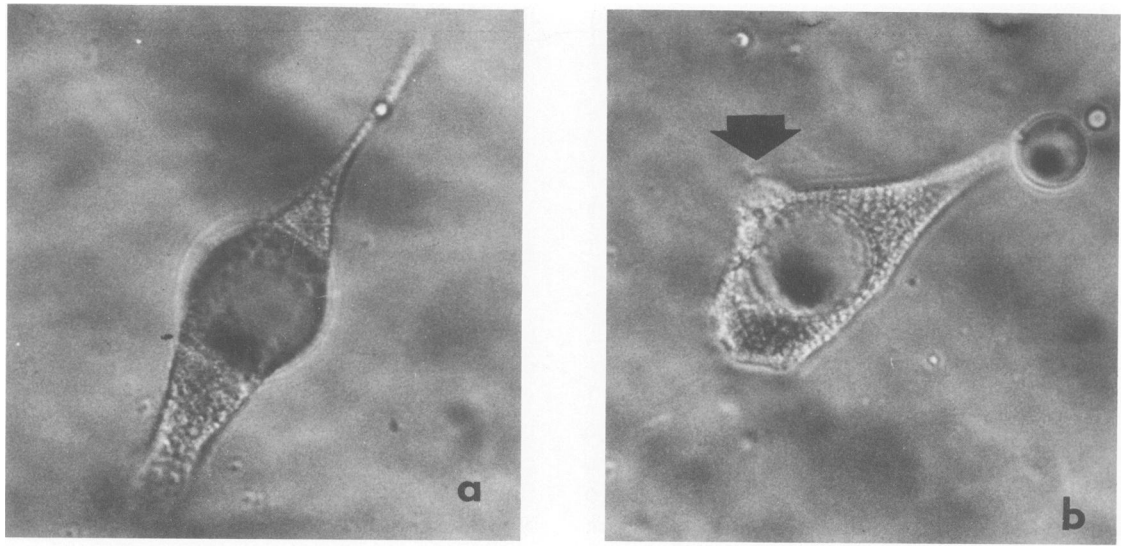


Figure 1a – A differentiated VSMC prior to ATII administration. Phase microscopy. (× 1000) **b** – A contracting differentiated VSMC after 6 minutes of ATII administration. Notice the characteristic perinuclear evagination (*arrow*). (× 1000)

Evaluation of SP/VSMC Interactions *in Vitro*

Analysis of the Interaction of MRL/lpr or C_3H SPs With MRL/lpr VSMCs

The next step was to determine whether lymphocytes from AI, but not normal, mice would initiate injury to VSMCs derived from mice with clinical vasculitis. One million SPs from either MRL/lpr or C_3H mice were cocultivated with MRL/lpr VSMCs. The SP/VSMC interactions were examined at 2-hour intervals up to 24 hours by light, phase, and scanning electron microscopy.

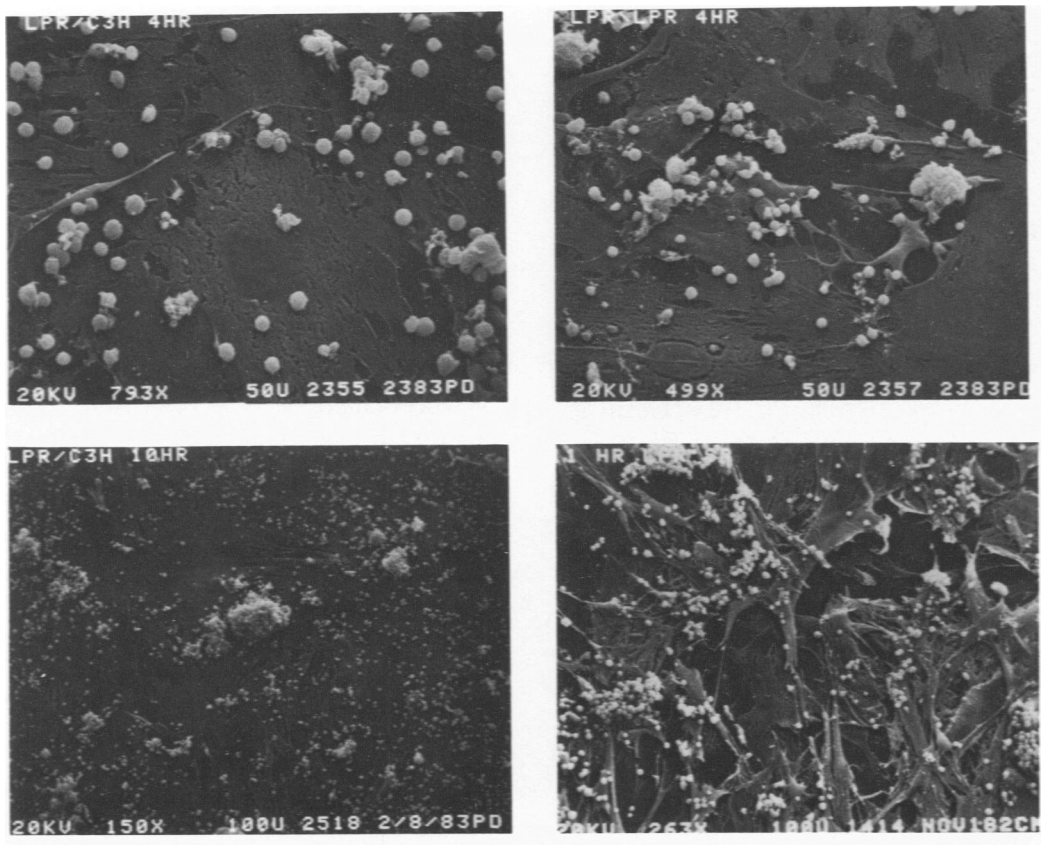
The intercellular interactions between C_3H and MRL/lpr SP populations with MRL/lpr VSMCs were characterized as follows: The earliest SP adherence to the culture plate was noted at 2 hours. Between 2 and 5 hours, both MRL/lpr and C_3H SP interactions were characterized as random, and minimal interleukocytic contact was observed. Between 5 and 14 hours, there were massive MRL/lpr and C_3H lymphocyte and macrophage cell clusters enveloping selected MRL/lpr VSMCs – an interaction which resulted in the release of MRL/lpr VSMCs from the culture plate. These interactions are summarized in Figure 2. This figure shows examples of splenocyte interactions at 4 hours (the top horizontal row) and at 10–11 hours (the bottom horizontal row). This comparison shows that both populations of SPs, regardless of whether they are derived from AI or normal mice, respond similarly; that is, they *both* exhibited massive envelopment of MRL/lpr between 5–14 hours. By 16 hours, all intercellular interactions had ceased.

An example of the mean number of MRL/lpr or C_3H SPs per MRL/lpr for each 2-hour time interval between 2 and 14 hours are displayed beneath the visual synopsis. The numbers range between 0.8 and 2.3 C_3H splenocytes/MRL/lpr VSMC and 0.5–8.3 MRL/lpr SPs/MRL/lpr VSMC. Between 6 and 14 hours, the MRL/lpr SP response was approximately two to six times increased over the C_3H SP response. Analysis of the SP values by ANOVA did not statistically substantiate this difference, therefore indicating that quantitatively both C_3H and MRL/lpr SPs adhered similarly to MRL/lpr VSMCs (CI > 95%). All four MRL/lpr VSMC experiments showed similar results.

Close examination of SP/VSMC interactions confirmed that a 5–7-μ, smooth to microvillar cell, consistent with lymphocyte morphology, was the primary cell establishing initial and continued contact with MRL/lpr VSMCs (Figure 3). Ten to twenty-micron pleomorphic cells with microprojections consistent with macrophage topography followed initial lymphocyte contact. Massive SP envelopment of MRL/lpr VSMCs did not involve each VSMC but was selective, as illustrated in the bottom row of Figure 2.

The electron photomicrographic data show that both C_3H and MRL/lpr SPs cluster around and envelop VSMCs derived from autoimmune mice. At the end of 14 hours, the VSMCs detach from the surface of the culture plate. Quantitatively, the number of C_3H versus MRL/lpr SPs/VSMC is statistically similar.

We next evaluated the interaction of C_3H or MRL/lpr SPs with normal (C_3H) VSMCs to determine whether similar or different conclusions could be drawn: ie, does the interaction of normal VSMCs with either SP population (as above) result in similar envelopment and destruction of *these* VSMCs?



C_3H or MRL/lpr Splenocytes/MRL/lpr VSMCs

	$\bar{X}$ SPs/VSMCs	
Hours	C_3H SPs	MRL/lpr SPs
2	0	0.5 ± 1.38
4	0.9 ± 1.15	0.9 ± 2.52
6	2.3 ± 2.56	8.3 ± 8.12
8	0.9 ± 1.14	5.6 ± 4.23
10	0.7 ± 0.85	6.3 ± 4
12	1.4 ± 1.79	3.6 ± 3.73
14	0.8 ± 1.08	1.9 ± 3.49

Figure 2—A comparative synopsis of splenocyte interactions with MRL/lpr VSMCs. C_3H splenocyte interactions are represented by the left vertical row. The right vertical row represents the MRL/lpr splenocyte responses. The top horizontal row is an example of splenocyte random adherence to the MRL/lpr VSMC monolayer at 4 hours. The bottom horizontal row exhibits examples of splenocyte clustering around MRL/lpr VSMCs at 10–11 hours. Notice that *both* splenocyte populations aggregate around and envelop MRL/lpr VSMCs. The mean C_3H and MRL/lpr SPs per MRL/lpr VSMCs are shown below the photomicrographs for quantitation of the SP-VSMC interactions.

Analysis of MRL/lpr Versus C_3H Splenocyte Interactions With C_3H VSMCs

One million C_3H or MRL/lpr SPs were cocultivated with C_3H VSMCs for 0–16 hours under identical conditions as outlined in A above.

Figure 4 is a comparative visual synopsis of the interactions between C_3H or MRL/lpr SPs and C_3H VSMCs. C_3H SP interactions are exhibited on the left vertical row, and MRL/lpr SP interactions are shown on the right vertical row. The top horizontal row shows SP/VSMC interactions at 4–4½ hours, and the bottom

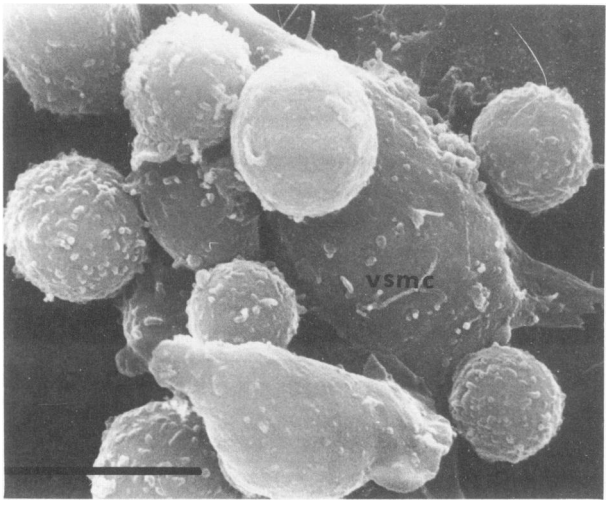


Figure 3 – Scanning electron photomicrograph of the lymphocyte clustering around a differentiated VSMC. The bar represents 5 μ, 30 kv.

horizontal row shows interaction at 14 hours. *Both* SP populations interacted with C_3H VSMCs in a random pattern with minimal interleukocytic contact. This lack of interaction did not change with time, as indicated by the 14-hour electron photomicrographs.

An example of the mean number of C_3H or MRL/lpr SPs per C_3H VSMC for each 2-hour time period are displayed beneath the visual synopsis. The numbers range between 7.1 and 11.1 C_3H SPs per C_3H VSMCs and 2.2 and 9.0 MRL/lpr SPs per C_3H VSMCs. Between 2 and 16 hours, *no* difference in the number of MRL/lpr or C_3H SP/VSMCs was determined when either SP population was cocultivated with C_3H VSMCs (CI > 95%).

These data indicated that, in direct contrast to MRL/lpr VSMCs, C_3H VSMCs did not induce SPs, regardless of their derivations, to envelop and destroy them. Quantitatively, there was no difference in the number of C_3H versus MRL/lpr SPs per MRL/lpr VSMC.

A comparison of the results of the quantitative and electron photomicrographic data suggests that MRL/lpr VSMCs induced a splenocyte response which differed from the response by the same populations of splenocytes to C_3H VSMCs. When MRL/lpr and C_3H VSMCs were evaluated statistically on the basis of the composite adherence of both (C_3H + MRL/lpr) splenocyte responses to each of these VSMCs, a significant difference was detected by two-way ANOVA (CI > 95%).

These observations raised the possibility that MRL/lpr VSMCs induced the mononuclear inflammatory cell influx. The next step was to determine potential mechanisms by which MRL/lpr VSMCs might stimulate splenocytes. Two functions were examined: the release of interleukin-1-like factor(s) and the expression of Ia.

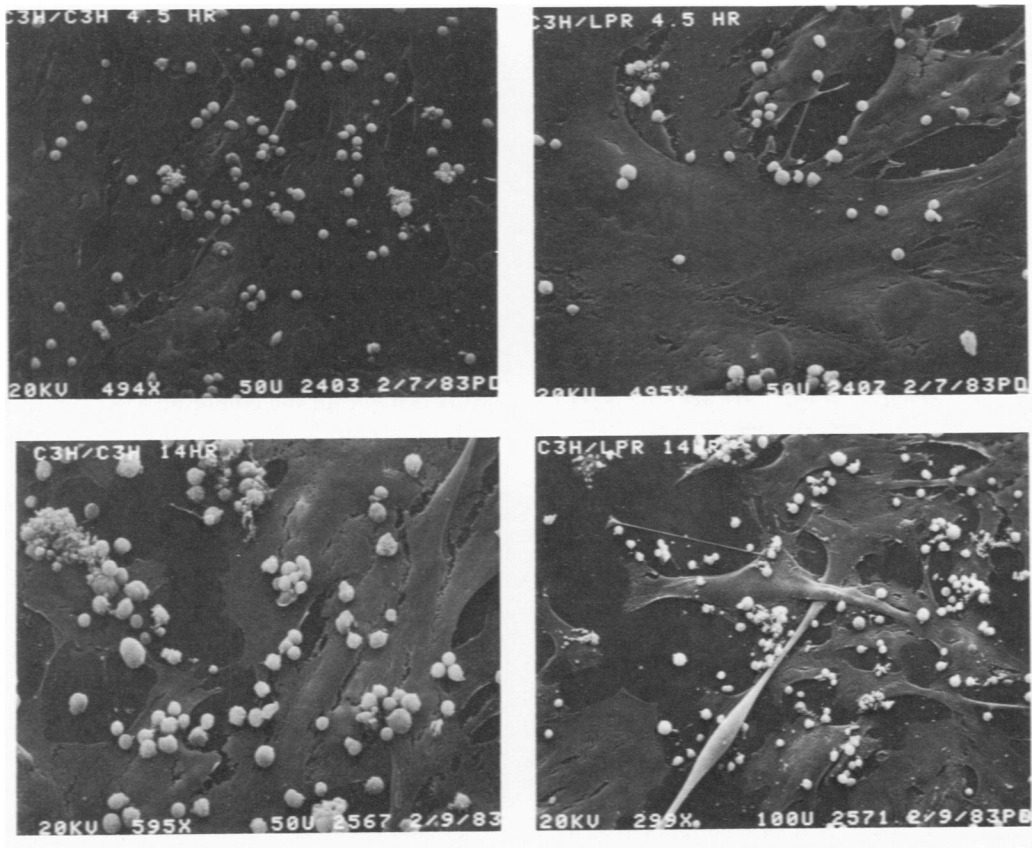
Evaluation of MRL/lpr VSMC Production of Interleukin-1-Like Factor(s)

The possibility that VSMCs from MRL/lpr mice might secrete factor(s) which would cause thymocytes to proliferate was examined. Supernatants from primary, and first through third passage, MRL/lpr and C_3H VSMCs were assessed. Figure 5 shows that, in the presence of mitogen, "80% supernatants" from primary, and first through third passage, MRL/lpr cultures increased thymocyte proliferation from less than 200 cpm to 21,156.8 ± 322, 15,083.6 ± 673.4, 17,566.6 ± 410.5, and 20,492 ± 1200.4, respectively. In contrast, supernatants derived from first through third passage C_3H VSMCs failed to enhance mitogen-dependent thymocyte proliferation. Primary C_3H VSMC cultures increased thymocyte proliferation from 141.6 ± 96.1 cpm to 12,780.8 ± 897.8 cpm. This thymocyte proliferation was probably due to macrophages, which are frequently present in primary cultures and are known to release 1L-1.[22,23] By subculture, these cells were eliminated. The experiment was repeated three times in triplicate with similar results. Therefore, mitogen-dependent thymocyte proliferation was enhanced following incubation with supernatants from VSMCs derived from autoimmune, but not normal, mice.

Evaluation of Ia Expression by MRL/lpr Vascular Smooth Muscle Cells

The expression of Ia by MRL/lpr and C_3H VSMCs was evaluated by two indirect immunofluorescence methods. Since C_3H and MRL/lpr are syngeneic at the H-2 histocompatability locus (H-2k), VSMCs, should they spontaneously express Ia, would express Ia^k. Figure 6 shows that MRL/lpr VSMCs by fluorescence-microscopic analysis spontaneously express Ia^k in a distinct granular and clustered pattern. In contrast, examination of C_3H VSMCs by fluorescence microscopy for Ia expression showed only a very weak, generalized background fluorescence, principally restricted to the nuclear membrane and the nucleolus. Indirect immunofluorescence staining using IgG2a as a negative control produced minimal nonspecific perinuclear immunofluorescence expression by both MRL/lpr and C_3H VSMCs.

These microscopic analyses suggested that Ia^k was expressed by MRL/lpr VSMCs. We next compared the level of Ia^k expression by C_3H versus MRL/lpr VSMCs using a flow cytometer. Figure 7 is a set of histograms



C_3H or MRL/lpr Splenocytes/C_3H VSMCs

	$\bar{X}$ SPs/VSMCs	
Hours	C_3H SPs	MRL/lpr SPs
2	10.8 ± 6.73	2.2 ± 2.07
4	7.1 ± 5.3	5.8 ± 4.63
6	10.0 ± 6.91	9.0 ± 7.22
8	10.6 ± 10.12	4.2 ± 3.27
10	11.1 ± 7.21	8.6 ± 5.5
12	7.7 ± 7.91	5.5 ± 4.18
14	9.8 ± 6.71	7.8 ± 1.22

Figure 4—A comparative synopsis of splenocyte interactions with C_3H VSMCs. The left vertical row represents the C_3H splenocyte responses, and the right vertical row represents the MRL/lpr splenocyte responses. The top horizontal row shows examples of MRL/lpr and C_3H splenocytes with C_3H VSMCs at 4 hours. The bottom horizontal row shows examples of MRL/lpr and C_3H splenocyte interactions with C_3H VSMCs at 10–14 hours. Notice that there is minimal leukocytic aggregation by *both* C_3H and MRL/lpr SP populations. The mean C_3H and MRL/lpr SPs per C_3H VSMC are shown below the photomicrographs for quantitation of the SP VSMC interactions.

illustrating Ia^k expression by C_3H VSMCs on the left and MRL/lpr VSMCs on the right. These histograms show that C_3H VSMCs exhibit normal background fluorescence only, as demonstrated by the single peak of fluorescence defined by the region in Channels 0–30, and do not express the Ia^k antigen. In contrast, a small peak of normal background fluorescence is expressed by the MRL/lpr VSMCs (region between Channels 0 and 30), but the Ia^k antigen is also expressed, indicated by the second peak of fluorescence in the region between Channels 31 and 180.

These findings indicate that MRL/lpr VSMCs express

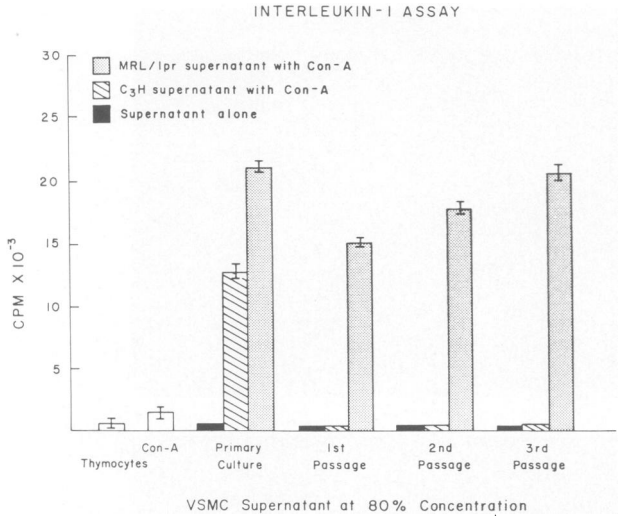


Figure 5 – Proliferative response of 1.5 × 10^6 thymocytes ± primary, and first through third passage, supernatants derived from MRL/lpr and C_3H VSMCs ± Con-A.

significantly higher levels of Ia^k than are expressed by C_3H VSMCs.

Discussion

The purpose of the present study was to define the role of VSMCs in AI vasculitis. Vascular smooth muscle cell cultures were derived from 1) MRL/lpr mice, which develop mononuclear cell vasculitis by 16 weeks, and 2) C_3H/Hej mice, which do not. The homogeneity of VSMCs in culture was confirmed both morphologically and functionally. Cocultivation studies indicated that MRL/lpr, but not C_3H, VSMCs induced lymphocytes and macrophages to cluster around and to envelop their cell surfaces. Progressive mononuclear cell aggregation ultimately resulted in the detachment of MRL/lpr VSMCs from the culture dish. Finally, MRL/lpr VSMCs spontaneously released interleukin-1-like factor(s) and expressed Ia, two mechanisms by which MRL/lpr VSMCs may have stimulated lymphocyte function.

Our interpretation of the interaction of splenocyte populations with normal versus AI VSMCs is the following: The electron photomicrographs showed that SP/VSMC interactions differed dramatically and were directly dependent on the derivation of the VSMC, not on the splenocyte. Likewise, there was a significant difference in the adherence of SPs to VSMCs, which was again dependent on the derivation of the VSMC. Although there was slightly greater clustering of MRL/lpr SPs than of C_3H SPs to MRL/lpr VSMCs, this difference was not statistically significant. Therefore, our data suggest that the *initiation* of autoimmune vasculitis is not attributable to an intrinsic defect in lymphoid cells, but resides at the VSMC level. However, what remain unclear are: 1) the sequence of events which occur after initial lymphoid cell contact is made, 2) which lymphoid subpopulation is involved with each sequential event, and 3) what functional differences exist between lymphoid cells derived from autoimmune versus normal animals in this model.

There is indirect evidence which supports the concept that the Ly 1+ T cell is the primary responding cell.[18-21] Numerous studies clearly demonstrate that the Ly 1+ inducer T-cell subset is the predominant proliferating cell within the enlarging MRL/lpr node.[18-21] Indirect immunofluorescence (data not shown) of *in vivo* mononuclear cell vasculitis indicates that this same subset of cells is the predominant lymphocyte population at the site of vasculitis.

Particular research effort in our laboratories will focus first on the question of whether the Qa1+ or Qa1− subset of Ly 1+ inducer cells responds to the Ia expressed by the autoimmune VSMC. The Ly 1+ T cell is, by extensive fluorescent activated cell sorter analysis in our laboratory, Qa1 − . However, the Qa1+ set may primarily respond to Class II antigens (reviewed by Dutton and Swain[24]), leaving open the question of the respective roles of Qa1+ and Qa1− Ly 1+ T-cell subsets in this lymphoproliferative disease.

Since mononuclear cell vasculitis is an acquired lesion, our findings imply that the regulation of both Ia expression and the synthesis of interleukin-1 like factor(s) by MRL/lpr VSMCs is abnormal.

Spontaneous Ia expression by MRL/lpr VSMCs raises numerous theoretical questions. Recent data show that monocyte clones isolated from MRL/lpr mice present antigen to Ia restricted antigen-specific T-cell hybridomas.[25] Interestingly, these monocyte clones inappropriately express Ia, which raises the possibility that

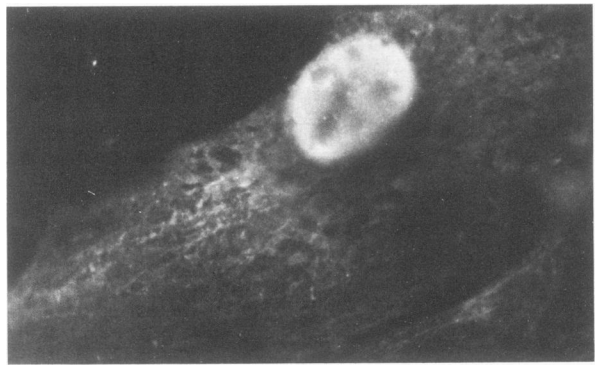


Figure 6 – A photomicrograph of indirect immunofluorescent studies showing Ia^k expression by MRL/lpr VSMCs. (Oil immersion, × 1000) Notice the distinct cytoplasmic granular clusters of fluorescence. The nucleus is out of the plane of focus so that the cytoplasmic Ia^k fluorescent staining is emphasized.

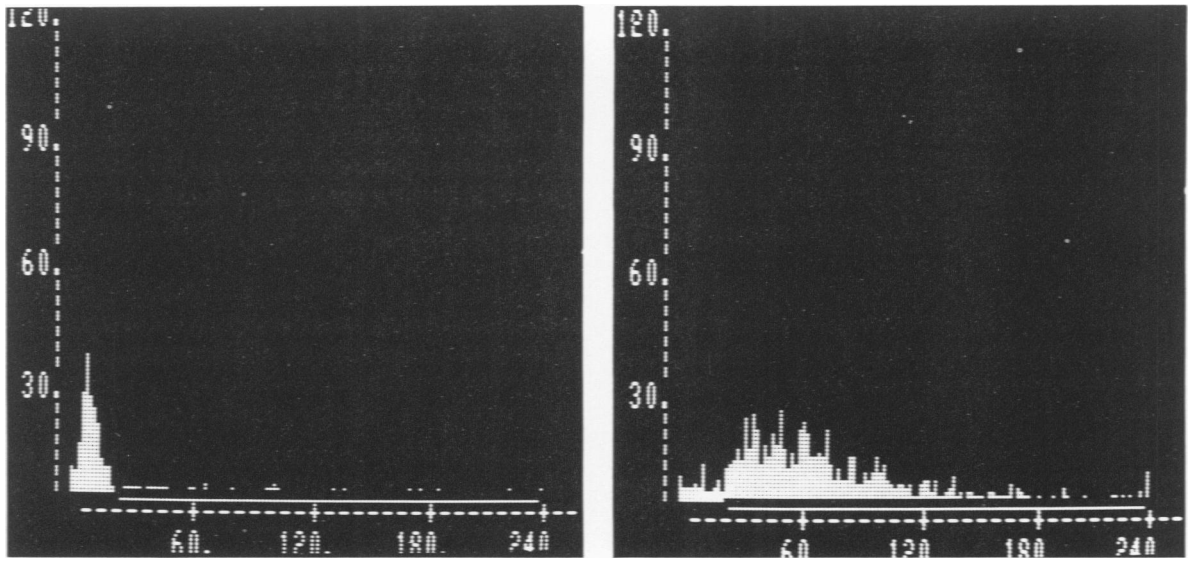


Figure 7 – Histograms demonstrating the expression of Ia^k by C_3H VSMCs (*left*) and MRL/lpr VSMCs (*right*). Note that C_3H VSMCs express only normal background fluorescence, as defined by the region in Channels 0–30. In contrast, MRL/lpr VSMCs express not only background fluorescence (Channels 0–30), but also Ia^k, as indicated by the peak of fluorescence defined by the region in Channels 31 – 180.

multiple Ia specificities may be expressed by MRL/lpr VSMCs. Whether or not VSMCs have functional and antigenic properties similar to those of the monocyte clones is under active investigation in our laboratories. The consequences of these dysregulations are also unclear, although ultimately the VSMC is probably destroyed as a result of its abnormal function. Thus, it is essentially a cell which initiates its own destruction.

These studies have raised numerous questions concerning the mechanisms of cell-mediated hypersensitivity in the production of autoimmune mononuclear cell vasculitis. Our current working hypothesis is that the VSMC from MRL/lpr mice initiates a splenocyte influx which culminates in autoimmune VSMC destruction. We have constructed a hypothetical mechanism, which may be occurring *in vivo*: 1) Initial randomized splenocyte migration progresses to 2) splenocyte migration to the VSMC site. This event may represent activation of sensitized T cells by Ia and "self" antigen, and 3) increased lymphoid influx occurs as the result of Il-1 like factor release. 4) Amplification of the lymphoid response occurs as a result of continued VSMC Ia expression and, perhaps, excessive Il-1 factor release. Furthermore, lymphokine release from responder T cells synergistically amplifies the response by recruiting additional lymphocytes and macrophages. Macrophages functionally amplify the T cell/VSMC interactions by creating improved lymphocyte access to VSMCs by releasing neutral proteases which break down connective tissue (response more evident *in vivo*). 5) Lymphocyte and macrophage envelopment of VSMCs culminates the VSMC autodestruction.

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Acknowledgments

We would like to thank Ms. Pat Dennis and Pat Cronin for their technical assistance, Ms. Barbara Berk for her assistance in preparation of the manuscript, Mr. Dave Cummings of the Whitaker College Laboratory of Interactive Microscopy at MIT (LIMMIT), and Dr. Robert C. Wolley, Cambridge Research Laboratory, Cambridge, Massachusetts, for his invaluable expert assistance with the flow-cytometry analyses. Finally, we would like to thank Dr. Robert McCluskey for his review of the manuscript and Dr. Walter Sheldon for his inspiration.