TWO MONOKINES, INTERLEUKIN 1 AND TUMOR NECROSIS FACTOR, RENDER CULTURED VASCULAR ENDOTHELIAL CELLS SUSCEPTIBLE TO LYSIS BY ANTIBODIES CIRCULATING DURING KAWASAKI SYNDROME

By DONALD Y. M. LEUNG, RAIF S. GEHA, JANE W. NEWBURGER, JANE C. BURNS, WALTER FIERS, LYNNE A. LAPIERRE, and JORDAN S. POBER

From the Departments of Pediatrics and Pathology, Harvard Medical School; the Divisions of Allergy, Cardiology and Infectious Disease, The Children's Hospital; the Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts 02115; and the Laboratory of Molecular Biology, the State University of Ghent, Ghent, Belgium

Kawasaki syndrome (KS)[1] is an acute illness of early childhood characterized by prolonged fever (5 d or longer), diffuse mucosal inflammation, indurative edema of the hands and feet, polymorphous skin rash, and nonsuppurative lymphadenopathy (1, 2). This disease has aroused much interest because of its association with sudden death stemming from coronary arteritis accompanied by coronary aneurysms and thrombotic occlusion (3, 4). The histopathologic findings in KS indicate a panvasculitis with endothelial necrosis, immunoglobulin deposition, and the infiltration of mononuclear cells into small- and medium-sized blood vessels (5, 6). Although much attention has been focused on the occurrence of coronary artery disease in KS, inflammation of other medium sized arteries also occurs (7, 8) and venulitis is a prominent finding in studies of autopsy cases (8).

The acute phase of KS is characterized by marked T cell and B cell activation (9, 10). The antibody repertoire that is activated in these patients, however, is poorly defined. Sera from patients with acute KS do not contain the usual autoantibodies frequently associated with collagen/vascular diseases, i.e., no rheumatoid factor, antinuclear, or anti-DNA antibodies have been detected (11). We have recently demonstrated that sera from patients with acute KS contain IgM antibodies that lyse cultured human umbilical vein endothelial (HUVE) and

This work was supported by grants AI-22058, AI-20373,HL-36003, and HL-22602 from the U. S. Public Health Service and by the National Foundation, March of Dimes. Research on tumor necrosis factor is supported by Biogen. Dr. Leung is the recipient of a New Investigator Research Award, 5R23 HL-30082, and Dr. Geha is the recipient of an Allergic Diseases Academic Award, K07 AI-0440, both from the National Institutes of Health. Dr. Pober is an Established Investigator of the American Heart Association.

[1] *Abbreviations used in this paper:* HEC, human endothelial cell; HDF, human dermal fibroblast; HSVE, human saphenous vein endothelial cell; HSVSM, human saphenous vein smooth muscle cell; HUVE, human umbilical vein endothelial cell; IC, immune complex; KS, Kawasaki syndrome; TNF, tumor necrosis factor.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/86/12/1958/15 $1.00
Volume 164 December 1986 1958–1972

human saphenous vein endothelial (HSVE) cells stimulated for 3–5 d with IFN-γ, a mediator secreted by activated T lymphocytes (12). Untreated HUVE cells were not lysed by sera from patients with acute KS. Previous observations have shown that IFN-γ can induce cultured human endothelial cells (HEC) to express class II major histocompatibility complex (MHC) antigens (13) and cause HEC to change shape and monolayer organization (14). These changes require 3–5 d of incubation for maximal effect.

The vascular lesion of acute KS is infiltrated with monocyte/macrophages as well as activated T lymphocytes. Activated monocyte/macrophages secrete the mediators IL-1 and tumor necrosis factor (TNF), which induce HEC to express tissue factor–like procoagulant activity (15, 16) and also induces HEC to become more adherent for leukocytes (17). Concomitant with these functional changes in HEC, we have recently demonstrated that TNF and IL-1 can induce HEC to express a new surface antigen detected by a monoclonal antibody, H4/18 (18). All these IL-1 or TNF effects are more rapid (peak effect in 4–6 h) than those mediated by IFN-γ, and are transient with loss of cell expression by 24 h, regardless of the continued presence or absence of monokines.

In the present study, we investigated the possibility that cytotoxic antibodies to IL-1- or TNF-inducible endothelial cell surface antigens could contribute to the vascular injury observed during the acute phase of KS. We report here that IgG and IgM antibodies in acute KS sera cause complement-mediated lysis of IL-1- or TNF-stimulated HUVE and HSVE cells but not control, IL-1-, or TNF-stimulated autologous human dermal fibroblasts (HDF) or human saphenous vein smooth muscle (HSVSM) cells. Furthermore, the target antigen(s) induced on endothelial cells by IL-1 or TNF differs from the previously described target antigen(s) induced by IFN-γ.

Materials and Methods

Cells. HUVE cells were isolated by the method of Gimbrone (19) and passaged under the conditions of Thornton et al. (20). Media, antibiotics, and tissue culture plastic (Corning) were all obtained from M.A. Bioproducts (Walkersville, MD); fetal calf serum was from Gibco (Grand Island, NY); endothelial cell growth supplement was from Meloy Laboratories Inc. (Springfield, VA); gelatin was from Difco Laboratories Inc. (Detroit, MI) and porcine heparin was purchased from Sigma Chemical Co. (St. Louis, MO). Cells were used upon serial subculture (passage levels 3–10). HDF were cultured from the foreskin of a donor whose umbilical cord was used to establish one of the HUVE cell cultures. HSVE cell and autologous HSVSM cell cultures were a gift of Peter Libby (Tufts University School of Medicine, Boston, MA).

Sera. A total of 31 samples of sera were obtained from 20 children with KS (12 boys, 8 girls; mean age, 2.1 yr; age range, 7 mo to 8 yr); 20 samples were obtained during the acute phase of KS, i.e., within 3 wk of the onset of fever, and 11 samples were obtained during the convalescent phase of KS (2–4 mo after resolution of acute symptoms). Seven patients were studied during both the acute and convalescent phases of their illness. The criteria used for diagnosis of KS were as previously described (12).

Sera were also obtained from 17 age-matched controls (10 boys, 7 girls; mean age, 2.6 yr; age range, 6 mo to 9 yr); five samples were obtained during evaluations for suspected allergic rhinitis in the Allergy Clinic at Children's Hospital, Boston, four samples were obtained during cardiac catheterization for evaluation of noninflammatory congenital heart disease, and eight samples were obtained from hospitalized patients with fever (temperature above 38.5°C) owing to bacterial illness (meningitis [$n = 4$], sepsis [$n = 2$], pyelonephritis [$n = 1$], and pneumonia [$n = 1$]).

In some experiments, sera from patients with acute KS were subjected to ultracentrifugation at 100,000 *g* for 1 h in an Airfuge (Beckman Instruments, Inc., Fullerton, CA) to remove any immune complexes present in the sera. In addition, control sera were heat-treated at 63°C for 1 h to form immune complexes (IC). The concentration of IC formed by heat treatment was quantitated by the Clq-binding assay (courtesy of Dr. P. Schur, Brigham and Women's Hospital, Boston). In selected experiments, acute KS sera were adsorbed with HUVE cells prestimulated with IL-1, TNF, IFN-γ, or control medium in order to define the nature of the target endothelial cell antigen. In these experiments, 0.6 ml of acute KS sera (diluted 1:2.5) was incubated with nonenzymatically suspended 1.5×10^6 control or mediator-treated HUVE cells for 1 h at 4°C before removing the cells by centrifugation.

Informed consent was obtained from each patient and/or his/her parents before performance of the study.

Isolation of Serum IgG and IgM. Monospecific affinity-purified anti-human IgG and anti-human IgM antibody was purchased from Tago Inc. (Burlingame, CA). These antisera were then coupled individually or in combination to CNBr-activated Sepharose 4B as outlined by the manufacturer (Pharmacia Fine Chemicals, Piscataway, NJ). To isolate serum IgG and IgM, acute KS sera was incubated with Sepharose 4B coupled with the appropriate antiserum for 2 h at 22°C on a rotator. The effluent (unbound material) from each suspension was then collected, the beads were washed extensively with ice-cold PBS, and the respective immunoglobulin isotype was eluted with 3 M sodium thiocyanate at 4°C. The eluates and effluents were immediately dialyzed against RPMI culture medium, adjusted to their original serum volumes, and stored at −80°C until tested. Purity of the IgG and IgM fractions were demonstrated by the Mancini immunodiffusion method (Kallestad Laboratories, Inc., Austen, TX).

Monoclonal Antibodies. The following murine monoclonal antibodies (mAb) were used during the course of this study: H4/18 (IgG1K, directed against an IL-1- or TNF-inducible surface endothelial cell antigen) (18), E1/1.2 (IgG2b, directed against monomorphic antigen on mesenchymal cells) (J. S. Pober and D. L. Mendrick, unpublished results), and LB3.1 (IgG2b, directed against an HLA-DR monomorphic determinant) (21).

Mediators. Human IL-1, affinity purified by the method of Dinarello from activated blood monocytes (22) and recombinant IL-1α (23) were purchased from Genzyme (Boston, MA). Recombinant IL-1β (24) was purchased from Cistron (Pinebrook, NJ). Human TNF was expressed from a cDNA clone in *Escherichia coli* (25) and was at least 99% pure. The endotoxin levels in these preparations were <1 ng/ml in the IL-1 stock solution (100 U/ml) and <10 ng/ml in the TNF stock solution (2.5×10^7 U/ml). Human IL-2, IFN-β, and IFN-γ were also used in the form of expressed recombinant products (26–29). IFN-α, expressed from a synthetic consensus sequence, was purchased from Amgen (Thousand Oaks, CA).

All of the preparations of mediators used were tested in bioassays, with HEC when appropriate. Specifically, monocyte-derived IL-1, recombinant IL-1α, recombinant IL-1β and recombinant TNF all induced HEC-specific expression of H4/18 binding (18). All of the interferons and TNF modulated HUVE cell expression of MHC antigens (13, 30). IL-2, which has no described action on HEC, was mitogenic for CTL line cells (31). None of the mediators at the concentrations or times used appeared toxic for the cultured cells as judged by loss of trypan blue dye exclusion or inhibition of radioactive amino acid incorporation into trichloroacetic acid–precipitable protein.

Treatment of Cell Cultures with Mediators. Mediators were added to various cell cultures in Medium 199, containing 20% FCS and antibiotics, at the following concentrations: IFN-γ, 200 U/ml; IL-1, 5 U/ml; TNF, 50 U/ml; IFN-α, 1,000 U/ml; IFN-β, 1,000 U/ml; and IL-2, 100 U/ml. Incubations continued for the times indicated in each experiment.

In endothelial cytotoxicity experiments, the effectiveness of IL-1 or TNF treatment was monitored by the induction of H4/18 mAb binding on replicate cultures using a radioimmunoassay (18). The appearance of cell surface class II MHC antigen was used to confirm that IFN-γ treatment was effective. The latter cell surface antigen was detected

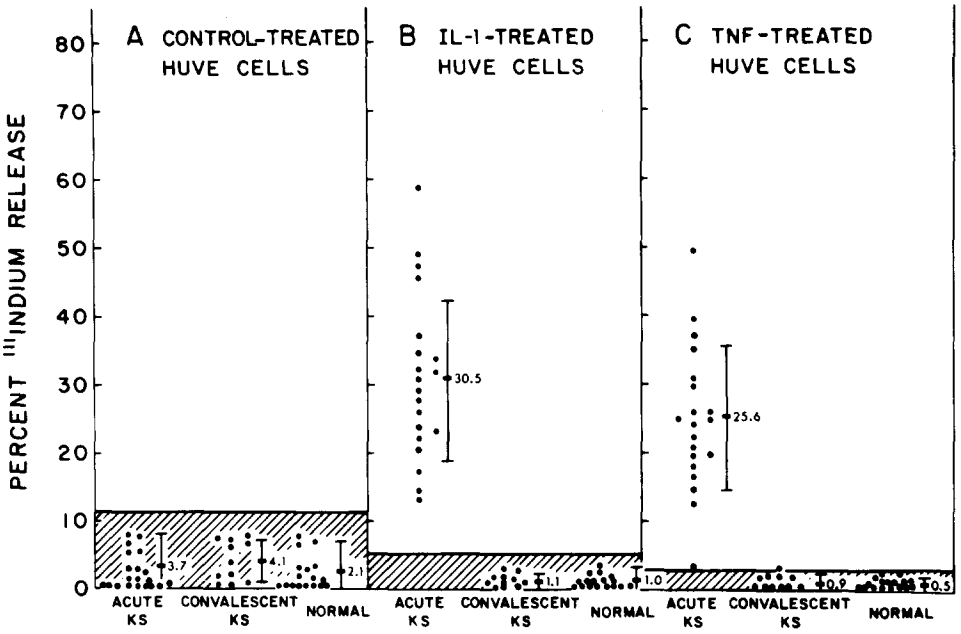

FIGURE 1. Cytotoxic effects of acute KS sera on cultured endothelial cells. Untreated (*A*), IL-1-treated (*B*) or TNF-treated (*C*) ^{111}In-labeled HUVE cells pretreated for 4 h with control medium (*A*), IL-1 (*B*), or TNF (*C*) were sequentially incubated with a 1:2.5 dilution of sera from patients with acute KS, convalescent KS, or normal controls, followed by a 1:1 dilution of rabbit serum. The percent specified ^{111}In release was determined. The mean ± 1 SD for each group is shown. Each solid circle is the mean result of an individual serum performed in triplicate. For sera tested on more than one occasion, the mean value for percent ^{111}In release obtained from these multiple determinations is shown.

both with an ^{111}In-release cytotoxicity assay using class II mAb (LB3.1) plus complement and by radioimmunoassay.

Detection of Cytotoxic Antibodies in Human Sera. An ^{111}In-release assay was used to detect cytotoxic anti-endothelial (HUVE or HSVE) cell, anti-smooth muscle (HSVSM) cell, or antifibroblast (HDF) antibodies in KS patient or control sera as previously described (12). Percent specific release (SR) was calculated as follows: (cpm experimental − cpm spontaneous)/(cpm total − cpm spontaneous). In each experiment, treatment with mAb E1/1.2 plus complement was used to establish maximal radioactivity that could be released. After cytolysis with mAb E1/1.2, the entire target cell population shows morphologically detectable damage, and the release of radioactivity into the culture medium is comparable to that obtained with detergent, e.g., NP-40, treatment.

Statistics. Comparison between the cytotoxic effects of patient and control sera were carried out using the nonparametric Wilcoxon rank-sum test. The results are expressed as the mean ± SD.

Results

Cytolysis of Vascular Endothelium Treated with IL-1, TNF, and Other Mediators by Sera from Patients with Acute KS. Sera from 20 patients in the acute phase of KS, 11 patients in the convalescent phase of KS, and 17 age-matched controls, 8 of whom had other febrile illnesses, failed to cause any significant ^{111}In release ($p > 0.2$) from untreated HUVE cultures (Fig. 1*A*). In contrast, sera obtained from the same group of patients with acute KS resulted in significantly higher

^{111}In release (30.5 ± 11.8; $p < 0.01$) from HUVE preincubated with monocyte-derived IL-1 for 4 h than did sera from age-matched controls (1.0 ± 2.1% ^{111}In release) as calculated by the Wilcoxon rank-sum test. All 20 acute KS sera caused ^{111}In release that was >2 SD above the mean value (i.e., > 5.2%) obtained using control sera (Fig. 1*B*). None of the 11 sera obtained from the convalescent phase of KS caused ^{111}In release greater than the normal range. The mean percent ^{111}In release caused by convalescent KS sera was not significantly different (1.1 ± 1.0; $p > 0.2$) than that obtained with normal sera and was significantly ($p < 0.1$) less than that caused by acute KS sera. Comparable susceptibility to lysis by acute KS sera could be induced with either recombinant human IL-1α or IL-1β polypeptides (results not shown).

TNF, like IL-1, has also been shown to induce new endothelial cell surface antigens within a 4-h incubation period (18). We, therefore, tested the cytotoxic effect of sera from the same three patient populations on endothelial cells pretreated with TNF for 4 h. The data in Fig. 1 *C* indicate that sera from patients with acute KS, but not sera from control patients or patients in the convalescent phase of KS, lysed TNF-stimulated HUVE ($p < 0.01$). The effects of IL-1 and TNF on HUVE appear specific because acute KS sera failed to lyse HUVE cells preincubated for 4 or 72 h with IFN-γ, IFN-β, or IL-2 (results not shown).

Characterization of Serum Cytotoxic Activity in KS. Sera from patients with acute KS were studied for the complement dependence of the cytotoxic effects on IL-1- or TNF-treated HUVE cells. In these experiments, ^{111}In-labeled IL-1 or TNF-treated HUVE cells were initially incubated with sera from patients with acute KS. Subsequently, fresh rabbit serum or heat-treated (56°C for 1 h) rabbit serum was added to the cultures. ^{111}In release was observed only when fresh rabbit serum, but not heat-inactivated rabbit serum, was added to IL-1- or TNF-treated HUVE cells preincubated with KS sera (results not shown).

Because circulating IC have been reported in patients with acute KS (32), we investigated the possibility that the observed complement-dependent cytotoxic effects of acute KS sera were being mediated via IC. The data in Table I indicates that, after centrifugation at 100,000 *g* to sediment IC, sera from acute KS patients continued to maintain the same level of antiendothelial cytotoxic activity as untreated sera. Furthermore, the formation of IC in normal sera by heat treatment (quantitated by Clq binding) was not associated with any detectable increase in endothelial cell cytotoxic activity.

The immunoglobulin class of cytotoxic anti–endothelial cell antibodies in acute KS was determined by passing serum from patients with acute KS through anti–human immunoglobulin–conjugated Sepahrose 4B columns. The results in Table II indicate that, when acute KS sera was absorbed with anti–human IgM Sepharose, both the effluent as well as the eluate fraction contained cytotoxic anti–endothelial cell activity. Similar results were obtained when acute KS sera was passed through anti–human IgG–conjugated columns. These data suggest that the cytotoxic antiendothelial activity in acute KS sera are associated with both the IgG and IgM antibody fraction. To exclude the possibility that a non-immunoglobulin fraction also mediated cytotoxicity, we passed acute KS sera over Sepharose conjugated with both anti–human IgG and anti–human IgM. As

TABLE I

Cytotoxic Effect of Acute KS Sera Is Not Mediated through Immune Complexes

Serum donor	Treatment	Percent specific ^{111}In release		
		Without mediator	With IL-1	With TNF
KS I	Untreated	0.7	32.1	32.9
	100,000 *g* centrifugation	0.6	31.6	31.7
KS II	Untreated	0	24.1	23.6
	100,000 *g* centrifugation	0	24.0	23.2
Normal	Untreated	0.2	0.4	0
	Heat treated	0	0	0

Sera from two patients with acute KS were centrifuged at 100,000 *g* for 1 h in a Beckman Airfuge, and the serum supernatants were compared with untreated sera for their ability to lyse ^{111}In-labeled untreated, IL-1-treated, or TNF-treated HUVE cells (4-h treatments). Serum from a normal donor containing <20 ng IgG IC/ml was incubated at 63°C for 1 h to form IC and compared with the untreated serum for its ability to lyse untreated HUVE cells or HUVE cells treated for 4 h with either IL-1 or TNF. After heat treatment, the normal serum contained 2,300 ng/ml of IgG IC as measured by the C1q binding assay. Data were calculated from means of triplicate determinations; all SD were <10%.

TABLE II

Cytotoxic Effects of Acute KS Sera on IL-1- or TNF-stimulated HUVE Cells Is Mediated through IgG and IgM Antibodies

Serum adsorption	IL-1-treated HUVE		TNF-treated HUVE	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Unadsorbed	25.9	45.3	33.5	64.7
Anti-IgG Sepharose				
Effluent	17.6	38.5	19.5	42.8
Eluate	8.9	17.2	18.8	27.6
Anti-IgM Sepharose				
Effluent	10.3	13.9	12.5	31.2
Eluate	22.2	44.1	30.8	40.6
Anti-IgG/anti-IgM Sepharose*				
Effluent	0	0	0.8	0
Eluate	39.8	66.2	42.6	51.0

Sera from six patients with acute KS (exp. 1 and exp. 2 each used a pool of sera from three donors) were fractionated by adsorption over anti-human IgG or anti-human IgM Sepharose or Sepharose conjugated with anti-human IgG and anti-human IgM. The effluent and eluate fractions of these columns were added to ^{111}In-labeled HUVE cell cultures pretreated with either IL-1 or TNF for 4 h, and ^{111}In release was measured after treatment with rabbit complement as described in Materials and Methods.

* These data were collected from a separate assay using the same pooled sera. Data were expressed as percent specific ^{111}In release and calculated from the mean of triplicate determinations; all SD were <10%.

TABLE III

Acute KS Sera Lyse IL-1- or TNF-treated HUVE Cells But Not IL-1- or TNF-treated Dermal Fibroblasts (HDF) Derived from the Same Donor

Target cells	KS sera donor	Percent specific ^{111}In release		
		Without mediator	With IL-1	With TNF
HUVE	1	0	35.6	37.8
HUVE	2	0	33.2	24.3
HUVE	3	0	28.3	29.8
HDF	1	1.3	1.1	0
HDF	2	1.4	0	0
HDF	3	1.4	0.8	1.1

Sera from three different patients with acute KS were tested for complement-dependent cytotoxic effects on HUVE cells and HDF derived from the same donor. Both cell types were pretreated for 4 h with control medium or medium containing IL-1 or TNF. Data were calculated as the means of triplicate determinations; all SD were <10%.

shown in Table II, the cytotoxic activity was restricted to the IgG and IgM fraction of acute KS sera.

Antibodies in Acute KS Lyse IL-1- or TNF-stimulated HUVE or HSVE Cells But Not IL-1- or TNF-stimulated HDF or HSVSM. The target specificity of cytotoxic anti–endothelial cell antibodies in acute KS was further examined by assessing the ability of sera from patients with acute KS to lyse control-treated, IL-1-treated, or TNF-treated HDF. As shown in Table III, three different acute KS sera were capable of lysing IL-1- or TNF-treated HUVE but failed to cause lysis of either control-treated, IL-1-treated, or TNF-treated HDF. These results were not due to allotypic differences that might occur between unrelated donors of HUVE cells and HDF because the HUVE cells and HDF used in Table III were derived from the same donor.

We further examined whether sera from acute KS were cytotoxic to HSVE vs. HSVSM cells. As shown in Table IV, four different acute KS sera lysed IL-1- or TNF-treated HSVE but not control-treated HSVE cells, control-treated HSVSM, IL-1-treated HSVSM, or TNF-treated HSVSM cells.

IL-1- or TNF-inducible Endothelial Target Antigen Is Rapidly But Only Transiently Expressed. Previous studies investigating mediator-induced synthesis of new endothelial cell proteins have found that IFN-γ requires 3–5 d for peak expression of class II MHC expression (13) whereas IL-1 or TNF only requires 4–6 h to maximally induce HEC to express procoagulant activity (15, 16), leukocyte adherence (17), or reactivity with the H4/18 mAb (18). We therefore examined the capacity of acute KS sera to lyse ^{111}In-labeled HUVE pretreated with control medium or medium containing IL-1, TNF, and IFN-γ for the following time periods: 4, 24, and 72 h.

The data shown in Table V indicate that HUVE become susceptible to lysis within 4 h of incubation with IL-1 or TNF. This effect, however, was not observed when the same HUVE were preincubated for 24 or 72 h with IL-1 or

TABLE IV

Acute KS Sera Lyse IL-1- or TNF-treated Endothelial Cells But Not IL-1- or TNF-treated Smooth Muscle Cells from Saphenous Vein of the Same Donor

Target cells	KS sera donor	Percent specific ^{111}In release		
		Without mediator	With IL-1	With TNF
HSVE	1	0.8	47.5	39.6
	2	0	29.3	19.6
	3	0.7	45.9	43.2
	4	1.1	28.7	33.6
HSVSM	1	1.1	0.7	1.4
	2	1.6	1.1	1.5
	3	0.8	2.0	2.6
	4	1.1	0	1.1

Sera from patients with acute KS were tested for complement-dependent cytotoxic effects on passaged HSVE and HSVSM cells derived from the same segment of the human saphenous vein. Both cell types were pretreated for 4 h with either control medium or medium containing IL-1 or TNF.

TABLE V

Time Course of Mediator-induced Endothelial Target Expression

HUVE treatment		Sera tested			
Duration (h)	Mediator	Control	KS 1	KS 2	KS 3
4	None	0	5.7	4.6	5.0
	IFN-γ	0	0	0	0
	IL-1	0	33.4	29.0	27.9
	TNF	0	29.2	31.0	25.8
24	None	4.4	5.2	2.6	2.7
	IFN-γ	0	4.9	6.2	8.7
	IL-1	0	3.4	3.7	3.2
	TNF	0	2.6	4.6	2.8
72	None	0	0	0	0
	IFN-γ	0	17.9	29.9	21.9
	IL-1	0	4.1	6.4	8.4
	TNF	0	4.9	8.9	5.0

Four sera, one from a control subject and three from patients with acute KS, were tested for their capacity to lyse HUVE cells treated with control medium or medium containing either IL-1, TNF, or IFN-γ for three time intervals: 4, 24, or 72 h. Data are expressed as percent specific ^{111}In release and calculated from the mean of triplicate determinations; all SD were <10%.

TNF. In contrast, HUVE required 72 h of incubation with IFN-γ before they became susceptible to lysis with acute KS sera. No significant lysis of HUVE was observed when they were preincubated with IFN-γ for 4 or 24 h.

TABLE VI

IL-1- or TNF-inducible Endothelial Target Antigen Recognized by Acute KS Sera Is Distinct from the IFN-γ-inducible Target Antigen

Adsorbent	HUVE target cell treatment			
	None	IFN-γ (3 d)	IL-1 (4 h)	TNF (4 h)
None	1.0	26.1	18.6	26.1
Control HUVE cells	0	18.4	19.2	20.9
IFN-γ-treated HUVE cells	0	0.7	25.3	30.0
IL-1-treated HUVE cells	0	13.6	3.6	4.4
TNF-treated HUVE cells	0	15.5	0	3.5

A pool of sera from three patients with acute KS was preincubated with either untreated HUVE cells, HUVE cells treated with IFN-γ for 72 h, HUVE cells treated with IL-1 for 4 h, or HUVE cells treated for 4 h with TNF, as described in Materials and Methods. Subsequently, these adsorbed sera were tested for their capacity to lyse ^{111}In-labeled HUVE cells treated either with control medium or HUVE cells stimulated with IFN-γ for 72 h, IL-1 for 4 h, or TNF for 4 h. Data are expressed as percent specific ^{111}In release and calculated from the mean of triplicate determinations; all SD were <10%.

Distinction of the IL-1- or TNF-inducible Target Endothelial Antigen from the IFN-γ-inducible Antigen. The different preincubation periods required by IFN-γ vs. IL-1 or TNF for rendering HUVE cells susceptible to lysis by acute KS sera strongly suggested that IFN-γ induced an endothelial cell target antigen(s) distinct from those induced by IL-1 or TNF. To compare further the nature of endothelial cell antigens induced by these three mediators, we tested the capacity of acute KS sera to lyse HUVE cells treated with IFN-γ for 72 h after adsorption with human control HUVE cells, IFN-γ-treated HUVE cells, HUVE cells treated for 4 h with IL-1, or HUVE cells treated for 4 h with TNF. The results in Table VI indicate that IFN-γ-treated HUVE cells effectively absorbed out cytotoxic anti–endothelial cell activity against IFN-γ-stimulated HUVE cells. In contrast, neither control-treated HUVE cells nor IL-1- or TNF-stimulated HUVE cells removed the cytotoxic activity of acute KS sera directed against IFN-γ-treated HUVE cells. Similarly, when acute KS sera were absorbed with IL-1 or TNF stimulated HUVE cells they were no longer cytotoxic to either IL-1- or TNF-treated HUVE cells. However, acute KS sera absorbed with IFN-γ-treated HUVE cells or control-treated HUVE cells continued to manifest cytotoxic activity against IL-1- or TNF-treated HUVE cells. The data in Table VI also indicate that IL-1 and TNF induce similar endothelial target antigens because absorption of acute KS sera with IL-1-treated HUVE cells removed the cytotoxic activity directed against TNF-treated HUVE cells and vice versa.

Discussion

Vascular endothelial cells are known to be targets for the actions of immune mediators. IL-1 was first noted to alter the surface of HEC by inducing expression of tissue factor–like procoagulant activity (15). IL-1 also increases HEC adhesiveness for polymorphonuclear leukocytes (17, 33), blood monocytes (17), and lymphocytes (34). IL-1 may additionally affect coagulation by increasing biosynthesis of prostacyclin (35) and platelet-activating factor (36), decreasing the generation of activated protein C (37), and increasing secretion of the specific

inhibitor of plasminogen activator (38–40). With the exception of prostacyclin synthesis, all of these actions would be expected to promote coagulation and inflammation. Furthermore, the generation of thrombin, through a tissue factor–activated procoagulant pathway, can cause HEC to release IL-1, potentially amplifying the inflammatory effects (41). TNF has been shown to act on HEC, increasing expression of class I MHC antigens (42) and causing HEC cultures to undergo a morphologic rearrangement that may facilitate transendothelial trafficking of leukocytes (14). Interesting, TNF shares a number of actions with IL-1, causing both an increase in HEC adhesiveness for leukocytes (43) and an induction of tissue factor–like procoagulant activity (16, 44). TNF also induces the release of IL-1 by HEC (45). The procoagulant and proinflammatory actions of TNF, by promoting vascular thrombosis and leukocytic infiltration, may contribute to the action of this mediator in causing hemorrhagic necrosis of tumors.

Many of the actions of IL-1 and TNF appear to involve modulation of HEC surface molecules. In fact, a monoclonal antibody raised to IL-1-stimulated HUVE cells detects a protein antigen the expression of which is inducible by treatment with IL-1 or with TNF (18). Previously, we had found that patients with KS had circulating antibodies directed to HEC antigens induced by IFN-γ. The present study was undertaken to test the hypothesis that antibodies directed to IL-1- or TNF-inducible endothelial cell surface antigens may contribute to the vascular damage observed during the acute phase of KS. Our current investigation demonstrates that IgG and IgM antibodies in sera from patients with acute KS lyse cultured HUVE or HSVE cells treated for 4 h with IL-1 or TNF but do not lyse control-treated HUVE or HSVE cells (Fig. 1). In contrast, sera from patients in the convalescent phase of KS, or age-matched controls, including eight patients with other febrile illnesses, failed to lyse either control-treated or monokine-treated endothelial cells. This complement-mediated cytotoxicity activity found in acute KS sera against vascular endothelial cells appears specific because these KS sera failed to lyse IL-1- or TNF-stimulated HDF or HSVSM cells autologous to the HEC. Preincubation of HUVE cells with other mediators such as IFN-α, IFN-β, IFN-γ, or IL-2 for 4 h failed to render HUVE susceptible to lysis with sera from acute KS.

In a recent study, we demonstrated that sera from patients with acute KS contain IgM antibodies cytotoxic to IFN-γ stimulated HUVE cells (12). The target endothelial antigen on IFN-γ-stimulated HUVE cells was not identified because these sera were unable to immunoprecipitate IFN-γ-induced HUVE cell proteins. However, it was found that acute KS sera could not lyse IFN-γ-stimulated HDF or vascular smooth muscle cells. Because IFN-γ induced the same level of class I or class II MHC antigen expression on HDF, smooth muscle cells, and endothelial cells, the target antigens recognized by acute KS sera were thought not to be MHC determinants. This conclusion was further strengthened by the observation that, when acute KS sera was absorbed with IFN-γ-treated HUVE cells, B cells, monocytes, T cells, or erythrocytes, only IFN-γ-treated HUVE cells removed the cytotoxic anti–endothelial cell activity.

The IL-1- or TNF-inducible target endothelial cell antigen(s) described in the current study appear to be distinct from the previously described IFN-γ-inducible

target antigen. This statement is supported by three observations. First, the time course for induction of target antigen expression by IL-1 or TNF differed from that seen for IFN-γ. The IL-1- or TNF-inducible effects on endothelial cells were rapid, occurring within 4–6 h, and transient, disappearing after 24 h of incubation with IL-1 or TNF, even in the continued presence of mediator. In contrast, the effects of IFN-γ were much slower, requiring 72 h to induce its effect on endothelial cells (Table V). These time intervals correlate with the optimal incubation periods required for the expression of other previously described inducible endothelial cell proteins by these mediators (18, 30). Second, antibodies reactive with IFN-γ-stimulated HUVE cells appeared to be exclusively of the IgM class (12), whereas some the antibodies reactive with IL-1- and TNF-stimulated cells are of the IgG class. The basis for this difference is unknown. Third, IFN-γ-treated HUVE cells but not IL-1-treated HUVE cells, TNF-treated HUVE cells, or control-treated HUVE cells were able to absorb out cytotoxic activity in acute KS were directed against IFN-γ-stimulated HUVE cells. The converse was also true: IL-1- or TNF-treated HUVE cells but not IFN-γ-treated HUVE cells effectively absorbed out cytotoxic activity in acute KS sera directed against IL-1- or TNF-stimulated HUVE cells.

In vivo, both types of antigens may be expressed at the same time. The highly immunogenic nature of vascular endothelial cells (reviewed in Pober et al. [46]) may contribute to the development of these autoantibodies. IL-1 and TNF seemed to induce similar endothelial cell target antigen(s) because IL-1-treated HUVE cells removed the cytotoxic activity in acute KS sera directed against TNF-stimulated HUVE cells and vice versa: absorption of acute KS sera with TNF-stimulated HUVE cells removed its cytotoxic activity against IL-1-stimulated HUVE cells. There is no possibility that these shared activities of IL-1 and TNF are due to mediator crosscontamination inasmuch as these effects are observed with recombinant monokines. These data extend the parallel actions of IL-1 and TNF on vascular endothelial cells previously noted (16, 18).

The reason for the disappearance of cytolytic antibodies to both IFN-γ or IL-1/TNF-induced antigens during the convalescent phase of KS is not known. It is possible that the decline in titer represents a fall in antibody level because of loss of antigenic stimulation (i.e., diminution of the expression of endothelial cell activation antigens), or active downregulation of antibody synthesis (e.g., suppression) or both. Alternatively, because our assay depends upon complement-mediated lysis, the appearance of antibodies that fail to fix complement, either by isotype switching or activation of different B cell clones, could lead to protective or "blocking" antibodies. Blocking antibodies could either mask the continued presence of cytolytic antibodies or, if of higher affinity, lead to inactivation of the original B cell populations.

The exact nature of the IL-1- or TNF-inducible endothelial target antigen(s) is presently unknown. Our attempts to immunoprecipitate IL-1- or TNF-inducible endothelial proteins with acute KS sera from HUVE cells metabolically labeled with [^{35}S]methionine have not, to date, been successful. The basis for this difficulty is not known but may be related to the poor antigen avidity of naturally occurring antibodies, or alternatively, because the antibodies in acute KS could be directed to inducible cell surface carbohydrate moieties that cannot

be labeled with radiolabeled amino acid precursors. These considerations are currently under investigation. In any event, the relevant antigenic determinants appear to be nonpolymorphic because KS sera have lysed HUVE and and HSVE cells from over five separate donors in the course of these experiments.

The observations made in our present study that patients with acute KS have circulating cytotoxic antibodies against IL-1- or TNF-stimulated endothelial cells, along with our previous report that sera from these patients contain antibodies directed against IFN-γ-inducible endothelial antigens, suggest the possibility that immune activation accompanied by the secretion of mediators are an important predisposing condition for the development of vascular damage. Indeed, we have recently examined PBMC from a small number of patients with acute KS for their capacity to secrete IL-1, as determined by the D10.G4.1 costimulator assay (47). PBMC from all three patients with acute KS studied but not age-matched control patients have been found to spontaneously secrete abnormally high levels of IL-1 (Leung and Pober, unpublished observations). Most vasculitides are characterized by polyclonal B cell activation and marked immune activation with the secretion of mediators. Thus, although our studies have focused exclusively on KS, we hypothesize that the production of cytotoxic antibodies directed to mediator-induced endothelial cell antigens may be a novel, previously unrecognized mechanism for vascular damage in other diseases as well.

Summary

Kawasaki syndrome (KS) is an acute febrile illness of early childhood characterized by diffuse vasculitis and marked immune activation. The present study was undertaken to determine whether the acute phase of KS is associated with circulating cytotoxic antibodies directed to target antigens induced on vascular endothelium by the monokines, IL-1, or tumor necrosis factor (TNF). Sera from 20 patients with acute KS, 11 patients in the convalescent phase of KS, and 17 age-matched controls were assessed for complement-dependent cytotoxic activity against ^{111}In-labeled human endothelial cells (HEC), dermal fibroblasts, and vascular smooth muscle cells. Sera from patients with acute KS but not the other subject groups caused significant ($p < 0.01$) complement-mediated killing of IL-1- or TNF-stimulated HEC. None of the sera tested had cytotoxicity against control HEC cultures or the other target cell types, with or without IL-1 or TNF pretreatment.

Expression of the IL-1- or TNF-inducible target antigens on endothelial cells was rapid and transient, peaking at 4 h and disappearing after 24 h despite continued incubation with monokine. In contrast, we have previously shown that IFN-γ requires 72 h to render HEC susceptible to lysis with acute KS sera. Serum adsorption studies demonstrated that IL-1- and TNF-inducible endothelial target antigens are distinct from IFN-γ-inducible antigens. These observations suggest that mediator secretion by activated monocyte/macrophages could be a predisposing factor to the development of vascular injury in acute KS. Although our present observations have been restricted to KS, the development of cytotoxic antibodies directed to monokine-inducible endothelial cell antigens may also be found in other vasculitides accompanied by immune activation.

The authors thank Dr. Michael Gimbrone for providing human umbilical vein endothelial cell cultures, Dr. Peter Libby for providing saphenous vein endothelial cells and smooth muscle cells, Dr. Donna Mendrick for monoclonal antibodies, Mr. Paul Cohill for technical assistance in cell culture, and Mr. David Lence and Mrs. Bonnie Snyder for their assistance in the preparation of this manuscript. We also thank Drs. Tucker Collins, Michael Gimbrone, and Ramzi Cotran for their helpful discussions.

Received for publication 12 June 1986 and in revised form 31 July 1986.

References

1. Kawasaki, T. 1967. Acute febrile mucocutaneous syndrome with lymphoid involvement with specific desquamation of the fingers and toes in children: Clinical observations of 50 cases. *Jpn. J. Allergol.* 16:178.
2. Yanagihara, R., and J. K. Todd. 1980. Acute febrile mucocutaneous lymph node syndrome. *Am. J. Dis. Child.* 134:603.
3. Kato, H., S. Koike, M. Yamamoto, Y. Ito, and E. Yano. 1975. Coronary aneurysms in infants and young children with acute febrile mucocutaneous lymph node syndrome. *J. Pediatr.* 86:892.
4. Kawasaki, T. 1971. Acute febrile mucocutaneous lymph node syndrome and sudden death. *Acta Paediatr. Jpn. (Overseas Ed.).* 75:433.
5. Hirose, S., and Y. Hamashima. 1978. Morphological observations on the vasculitis in the mucocutaneous lymph node syndrome. *Eur. J. Pediatr.* 129:17.
6. Landing, B. H., and E. J. Larson. 1977. Are infantile periarteritis nodosa and fatal mucocutaneous lymph node syndrome the same? *Pediatrics.* 59:651.
7. Tanaka, N., K. Sckimoto, and S. Naoe. 1976. Kawasaki disease: relationship with infantile periarteritis nodosa. *Arch. Pathol. Lab. Med.* 100:81.
8. Fujiwara, H., and Y. Hamashima. 1978. Pathology of the heart in Kawasaki's disease. *Pediatrics.* 61:100.
9. Leung, D. Y. M., R. L. Siegel, S. Grady, A. Krensky, R. Meade, E. L. Reinherz, and R. S. Geha. 1982. Immunoregulatory abnormalities in mucocutaneous lymph node syndrome. *Clin. Immunol. Immunopathol.* 23:100.
10. Leung, D. Y. M., E. T. Chu, N. Wood, S. Grady, R. Meade, and R. S. Geha. 1983. Immunoregulatory T cell abnormalities in mucocutaneous lymph node syndrome. *J. Immunol.* 130:2002.
11. Lee, L. A., J. Burns, M. Glode, C. Harmon, and W. Weston. 1983. No autoantibodies to nuclear antigens in Kawasaki syndrome. *N. Engl. J. Med.* 308:1034.
12. Leung, D. Y. M., T. Collins, L. A. Lapierre, and R. S. Geha. 1986. IgM antibodies present in the acute phase of Kawasaki syndrome lyse cultured vascular endothelial cells stimulated by gamma interferon. *J. Clin. Invest.* 77:1428.
13. Pober, J. S., M. A. Gimbrone, Jr., R. S. Cotran, C. S. Reiss, S. J. Burakoff, W. Fiers, and K. A. Ault. 1983. Ia expression by vascular endothelium is inducible by activated T cells and by human γ-interferon. *J. Exp. Med.* 157:1339.
14. Stolpen, A. H., E. C. Guinan, W. Fiers, and J. S. Pober. 1986. Recombinant tumor necrosis factor and immune interferon act singly and in combination to reorganize human vascular endothelial cell monolayers. *Am. J. Pathol.* 123:16.
15. Bevilacqua, M. P., J. S. Pober, G. R. Majeau, R. S. Cotran, and M. A. Gimbrone, Jr. 1984. Interleukin 1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. *J. Exp. Med.* 160:618.
16. Bevilacqua, M. P., J. S. Pober, G. R. Majeau, W. Fiers, R. S. Cotran, and M. A. Gimbrone, Jr. 1986. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. *Proc. Natl. Acad. Sci. USA.* 83:4533.

17. Bevilacqua, M. P., J. S. Pober, M. E. Wheeler, R. S. Cotran, and M. A. Gimbrone, Jr. 1985. Interleukin-1 acts on cultured human vascular endothelium to increase adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. *J. Clin. Invest.* 76:2003.
18. Pober, J. S., M. P. Bevilacqua, D. L. Mendrick, L. A. Lapierre, W. Fiers, and M. A. Gimbrone, Jr. 1986. Two distinct monokines, interleukin 1 and tumor necrosis factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. *J. Immunol.* 136:1680.
19. Gimbrone, M. A., Jr. 1976. Culture of vascular endothelium. *Prog. Hemostasis Thromb.* 3:1.
20. Thornton, S. C., S. N. Mueller, and E. M. Levine. 1983. Human endothelial cells: use of heparin in cloning and long-term serial cultivation. *Science (Wash. DC).* 222:623.
21. Muchmore, A. V., M. Megson, J. M. Decker, P. Knudsen, D. L. Mann, and S. Broder. 1983. Inhibitory activity of antibodies to human Ia-like determinants: Comparison of intact and pepsin-digested antibodies. *J. Immunol.* 131:725.
22. Rosenwasser, L. J., and C. A. Dinarello. 1981. Ability of human leukocyte pyrogen to enhance phytohemagglutinin induced murine thymocyte proliferation. *Cell. Immunol.* 63:134.
23. March, C. J., B. Mosley, A. Larsen, D. P. Ceretti, G. Braedt, V. Price, C. S. Henney, S. R. Kronheim, K. Grabstein, P. J. Conlon, T. P. Hopp, and D. Cosman. 1985. Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature (Lond.).* 315:641.
24. Auron, P. E., A. C. Webb, L. J. Rosenwasser, S. F. Mucci, A. Rich, S. M. Wolff, and C. A. Dinarello. 1984. Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. *Proc. Natl. Acad. Sci. USA.* 81:7907.
25. Marmenout, A., L. Fransen, J. Tavernier, J. Van der Hayden, R. Tizard, E. Kawashima, A. Shaw, M. J. Johnson, D. Simon, R. Muller, M.-R. Ruysschaert, A. Van Vliet, and W. Fiers. 1985. Molecular cloning and expression of human tumor necrosis factor and comparison with mouse tumor necrosis factor. *Eur. J. Biochem.* 152:512.
26. Devos, R., G. Plaetinck, H. Cheroutre, G. Simons, W. Degrave, J. Tavernier, E. Remaut, and W. Fiers. 1983. Molecular cloning of human interleukin 2 cDNA and its expression in *E. coli. Nucleic Acids Res.* 11:4307.
27. Derynck, R., J. Content, E. DeClercq, G. Volckaert, J. Tavernier, R. Devos, and W. Fiers. 1980. Isolation and structure of a human fibroblast interferon gene. *Nature (Lond.).* 285:542.
28. Fiers, W., E. Remaut, R. Devos, H. Cheroutre, R. Contreas, D. Geysen, W. DeGrave, P. Stanssens, J. Tavernier, Y. Taya, and J. Content. 1982. The human fibroblast and human immune interferon genes and their expression in homologous and heterologous cells. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 299:29.
29. Scahill, S. J., R. Devos, J. Vannder Heyden, and W. Fiers. 1983. Expression and characterization of the product of a human immune interferon cDNA gene in Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA.* 80:4654.
30. Pober, J. S., T. Collins, M. A. Gimbrone, Jr., R. S. Cotran, J. D. Gitlin, W. Fiers, C. Clayberger, A. M. Krensky, S. J. Burakoff, and C. S. Reiss. 1983. Lymphocytes recognize human vascular endothelial and dermal fibroblast Ia antigens induced by recombinant immune interferon. *Nature (Lond.).* 305:726.
31. Gillis, S., M. M. Ferm, W. Ou, and K. A. Smith. 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120:2027.
32. Mason, W. H., S. C. Jordan, R. Sakai, M. Takahashi, and B. Berstein. 1985. Circulating immune complexes in Kawasaki syndrome. *Pediatr. Infect. Dis.* 4:48.

33. Schleimer, R. P., and B. K. Rutledge. 1986. Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with interleukin 1, endotoxin and tumor-promoting phorbol diesters. *J. Immunol.* 136:649.
34. Cavender, D. E., D. O. Haskard, B. Joseph, and M. Ziff. 1986. Interleukin 1 increases the binding of human B and T lymphocytes to endothelial cell monolayers. *J. Immunol.* 136:203.
35. Rossi, V., F. Breviario, P. Ghezzi, E. Dejana, and A. Mantovani. 1985. Prostacyclin synthesis induced in vascular cells by interleukin-1. *Science (Wash. DC).* 229:174.
36. Bussolino, F., F. Breviario, C. Tetta, M. Agilietta, A. Mantovani, and E. Dejana. 1986. Interleukin 1 stimulates platelet-activating factor production in cultured human endothelial cells. *J. Clin. Invest.* 77:2027.
37. Nawroth, P., D. A. Handley, C. T. Esmon, and D. M. Stern. 1986. Interleukin 1 induces endothelial cell procoagulant while suppressing cell-surface anticoagulant activity. *Proc. Natl. Acad. Sci. USA.* 83:3460.
38. Emeis, J. J., and T. Kooistra. 1986. Interleukin 1 and lipopolysaccharide induce an inhibitor of tissue-type plasminogen activator in vivo and in cultured endothelial cells. *J. Exp. Med.* 163:1260.
39. Nachman, R. L., K. A. Hajjar, R. L. Silverstein, and C. A. Dinarello. 1986. Interleukin 1 induces endothelial cell synthesis of plasminogen activator inhibitor. *J. Exp. Med.* 163:1595.
40. Bevilacqua, M. P., R. R. Schleef, M. A. Gimbrone, Jr., and D. J. Loskutoff. 1986. Regulation of the fibrinolytic system of cultured human vascular endothelium by interleukin I. *J. Clin Invest.* 78:587.
41. Stern, D. M., I. Bank, P. P. Nawroth, J. Cassimeris, W. Kisiel, J. W. Fenton, C. Dinarello, L. Chess, and E. A. Jaffe. 1985. Self-regulation of procoagulant events on the endothelial cell surface. *J. Exp. Med.* 162:1223.
42. Collins, T., L. A. Lapierrre, W. Fiers, J. L. Strominger, and J. S. Pober. 1986. Recombinant tumor necrosis factor increases mRNA levels and surface expression of HLA-A,B antigens in vascular endothelial cells and dermal fibroblasts in vitro. *Proc. Natl. Acad. Sci. USA.* 83:446.
43. Gamble, J. R., J. M. Harlan, S. J. Klebanoff, and M. A. Vadas. 1985. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proc. Natl. Acad. Sci. USA.* 82:8667.
44. Nawroth, P. P., and D. M. Stern. 1986. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *J. Exp. Med.* 163:740.
45. Nawroth, P. P., I. Bank, D. Handley, J. Cassimeris, L. Chess, and D. Stern. 1986. Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. *J. Exp. Med.* 163:1363.
46. Pober, J. S., T. Collins, M. A. Gimbrone, Jr., P. Libby, and C. S. Reiss. 1986. Inducible expression of class II major histocompatibility complex antigens and the immunogenicity of vascular endothelium. *Transplantation.* 41:141.
47. Kaye, J., S. Parcelli, J. Tite, B. Jones, and C. A. Janeway. 1983. Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. *J. Exp. Med.* 158:836.