Kinetics of Acute Inflammation Induced by Escherichia coli *in Rabbits*

II. The Effect of Hyperimmunization, Complement Depletion, and Depletion of Leukocytes

MARGARET M. KOPANIAK, PhD, and HENRY Z. MOVAT, MD, PhD

From the Division of Experimental Pathology, Department of Pathology, University of Toronto, Medical Sciences Building, Toronto, Ontario, Canada

The inflammatory response to *Escherichia coli* was quantitated in the skin of normal rabbits and the kinetics established as described previously. Hyperemia, measured with radiolabeled microspheres; vascular permeability, estimated with ^{125}I-albumin; and leukocyte infiltration, quantitated with ^{51}Cr-labeled autologous leukocytes, reached maximal values 3 hours after the injection of bacteria and subsided almost completely by 6 hours. Hemorrhage, measured with homologous ^{59}Fe-erythrocytes, continued to increase between 1 and 6 hours after injection and then reached plateau levels. The lesions were studied up to 8 hours, since in the previous study no changes were observed beyond that time. In the study described in this paper, the host mediation systems were manipulated in various groups of rabbits in order to elucidate the mechanisms underlying the development of the inflammatory reaction. One group of animals was hyperimmunized with the *E coli* organisms, another was partially depleted of hemolytic complement with cobra venom factor, and yet another was rendered leukopenic with nitrogen mustard. In hyperimmunized animals hyperemia in the dermal lesions induced by the microorganisms was significantly more intense than in normal rabbits. Vascular permeability increase occurred earlier in hyperimmunized rabbits and at 1 hour was significantly greater than in normals. Decomplemented rabbits had significantly less vascular permeability than normal animals, whereas in leukopenic rabbits no increase in vascular permeability could be elicited. Leukocyte accumulation was increased over the normal animals in the lesions of hyperimmunized rabbits. Hemorrhage was significantly decreased in leukopenic rabbits. Histologic examination of the lesions revealed that whereas in normal animals the infiltrating neutrophils ingested most of the bacteria and formed definite abscesses by 6–8 hours, these abscesses were absent in leukopenic animals, and free-lying bacteria were demonstrable in lesions. Histologically more neutrophils were present in the hyperimmunized than in the normal rabbits, but this difference was striking when normal animals were compared with leukopenic animals, in some of which only very occasional small accumulations of neutrophils were present (Am J Pathol 1983; 110:13–29)

THE STUDY of the acute inflammatory reaction induced in the rabbit with *Escherichia coli* has established the kinetics of development of such vascular and cellular changes as hyperemia, edema (exudate) formation, and infiltration of neutrophils.[1] Infection with gram-negative bacteria has gained in clinical significance in recent years.[2] They have been shown to produce vascular injury, demonstrable as an increase in vascular permeability and hemorrhage. Gram-negative infection is particularly important because of the thrombohemorrhagic lesions induced by endotoxin, which is part of the bacterial cell wall.[3]

Supported by the Medical Research Council of Canada (MT-1251). This publication represents data which constitute Dr. Kopaniak's Dissertation and was submitted to the School of Graduate Studies, Toronto, in partial fulfillment of a Ph.D. program. Dr. Henry Z. Movat is a Career Investigator of the Medical Research Council of Canada.

Accepted for publication July 27, 1982.

Dr. Kopaniak's present address is Department of Pediatrics, National Jewish Hospital and Research Center, 3800 E. Colfax Avenue, Denver, CO 80206.

Address reprint requests to Dr. Henry Z. Movat, Division of Experimental Pathololgy, Department of Pathology, University of Toronto, Medical Sciences Building, Toronto, Ontario, Canada M5S 1A8.

0002-9440/83/0107-0013$01.65

The involvement of host mediation systems was of primary interest in this investigation. The neutrophil, due to its abundance at the inflammatory site,[1] appeared to play an important role in mediating the vascular phenomena and the ensuing vascular and tissue injury. Consequently, the host humoral systems known to modulate the activity of the neutrophils were manipulated, and eventually the neutrophils themselves were depleted, followed by the quantitation of the dermal lesions induced with *E coli*. The kinetics and extent of lesion development in rabbits hyperimmunized with the bacteria, depleted of hemolytic complement, and depleted of leukocytes were studied and compared with the kinetics and magnitude of the reaction in normal control rabbits.

Materials and Methods

Inflammatory Lesions

All experiments were performed on randomly bred female New Zealand albino rabbits (2.5–3.0 kg). *E coli* (Strain BAM, Medical Teaching Laboratories, University of Toronto, Toronto, Ontario) were grown in minimum nutrient broth, killed with 0.5% formaldehyde (4 C, overnight), and, following two washes, suspended in sterile saline at the concentration of 3×10^9 bacteria/ml, as described previously.[1] The hair on the back of rabbits was removed with electric clippers, and *E coli* were injected intradermally at 6×10^8 organisms per site in 0.2 ml sterile pyrogen-free saline. In order to obtain lesions of various ages at the time of sacrifice, the bacteria were administered at different times before the killing of the rabbits. After the rabbits were sacrificed, their dorsal skin was removed and frozen, and the lesions were punched out with a cork borer (1.5 cm in diameter). The punched-out skin disks were deposited in plastic vials for γ-spectrometric estimation of the amount of radioactivity accumulated in the lesions. Prior to freezing of the skin, blood was expressed manually out of the major vessels of the skin in all experiments, except those in which hyperemia was measured with microspheres. This massaging-out of blood reduced variation between control skin sites and decreased the background radioactivity.

Control Skin Sites

Sites checked for hyperemia 45 minutes after the intradermal injections of saline were found not to be significantly different from skin sites from animals that did not receive injections. Similarly, no such difference was found with respect to increased vascular permeability, accumulation of leukocytes, or hemorrhage in the experiments described in this paper. Thus, the results shown in the illustrations express the counts per minute (cpm) in the lesions over normal skin and normal saline-treated sites (NS).

In some experiments prostaglandin E_2 (PGE_2) was used as a positive control for hyperemia, as described before.[4] The PGE_2 was obtained from the Upjohn Co. (Kalamazoo, Mich), and the stock solutions made up in 95% ethanol were diluted before use with pyrogen-free saline. The rabbits received 1 μg/site 30 minutes before sacrifice.

Bradykinin-treated sites (Sigma Chemical Company, St. Louis, Mo) served as positive controls for vascular permeability in some experiments. Fifty nanograms of bradykinin was injected intradermally 15 minutes before sacrifice.

Measurement of Hyperemia

The radiolabeled microsphere localization method as described by Hay et al[5] was used for quantitation of hyperemia in the inflammatory sites. ^{57}Co-, ^{113}Sn-, or ^{46}Sc-labeled microspheres (10 or 15 μ in diameter, suspended in 10% dextran) obtained from New England Nuclear (Lachine, Quebec) were used. The microspheres (approximately 15 μCi per rabbit) were injected systemically via a catheter positioned in the right carotid artery of an anesthesized animal. The microspheres were infused 2–5 minutes before the killing of the animals, as was described previously.[1] The rabbits were sacrificed with an overdose of sodium nembutal. The γ-spectrometer (Intertechnique Canatech Inc., Toronto, Ontario) setting for ^{57}Co and ^{46}Sc was 70–400 keV; and for ^{113}Sn, which was measured by itself, a wide-open window was used. When data for microsphere trapping were pooled from several animals within a group, the skin values were expressed as a ratio of counts per minute per lesion to counts per minute per control site.

Measurement of Vascular Permeability

The increase of permeability of vessels to fluid and proteins was measured with the aid of ^{125}I-labeled human serum albumin (^{125}I-HSA), which was injected systemically via the carotid catheter or via the marginal ear vein, 15 minutes prior to sacrifice. Approximately 40 μCi of ^{125}I-HSA was injected per rabbit, as described before.[1] The amount of ^{125}I-HSA leaking out of blood vessels is directly proportional to the degree of enhancement in vascular permeability;[6] thus, this parameter was quantitated by measurement

of the amount of ^{125}I-radioactivity localized at the inflammatory sites. The window setting on the γ-spectrometer was adjusted to minimize the spill of emission from other isotopes into the iodine channel. However, when vascular permeability was measured with other parameters and ^{57}Co, ^{46}Sc, ^{113}Sn, ^{59}Fe, or ^{51}Cr were present, any interference from them was corrected for in the final calculation of results. The increase in permeability was initially expressed as a ratio of ^{125}I-counts per minute per lesion to counts per minute in normal skin. In later experiments equivalents of microliters of extravasated plasma were calculated by dividing the ^{125}I-cpm/lesion by the counts per minute/μl of plasma obtained after infusion of ^{125}I-HSA.

Measurement of Leukocyte Emigration

The accumulation of leukocytes in the inflammatory sites was measured by the technique of Issekutz and Movat.[7] Thirty milliliters of blood collected from the central ear artery of rabbits into 0.2% ethylene diaminetetraacetic acid (EDTA) were mixed with 7.5 ml of 1% hydroxyethyl cellulose (Polysciences, Inc., Warrington, Pa) and incubated at 37 C to sediment the erythrocytes. The leukocyte-rich plasma (LRP) was repeatedly removed for 30 minutes. The LRP was spun at 350*g* for 10 minutes at 4 C. The leukocytes, together with contaminating erythrocytes, were resuspended in Tyrode's solution free of Ca^{++} and Mg^{++} and containing 10% platelet-free heparinized plasma. One-hundred fifty microcuries of Na_2 $^{51}CrO_4$ (New England Nuclear, Lachine, Quebec) were addd to the cell suspension and incubated for 30 minutes at 37 C. Following the incubation the ^{51}Cr-leukocytes were washed in Ca^{++},Mg^{++}-free Tyrode's solution, centrifuged (350*g* for 10 minutes at 4 C), and reinfused into the marginal ear vein of the donor rabbit 40 minutes before the animal was killed, as described previously.[1] An aliquot (200 μl) of the labeled leukocyte suspension was taken before the cells were infused into the rabbits. The erythrocytes in the aliquot were lysed with 0.84% NH_4Cl in two consecutive incubations, and the total counts per minute infused on leukocytes were calculated from the counts per minute in the aliquot in which the erythrocytes had been lysed. The number of leukocytes infused was calculated from another aliquot, which was used for a hemocytometer count.

The radioactivity was counted on γ-spectrometer window setting of 70–400 keV. The percentage spill of energy emission from ^{59}Fe into the ^{51}Cr channel was subtracted for each sample. Leukocyte migration studies were never performed together with hyperemia measurements, because of technical difficulties. The accumulation of leukocytes was expressed as a ratio of ^{51}Cr counts per minute per lesion to counts per minute per normal skin.

In leukocyte labeling experiments all solutions were made up in sterile, pyrogen-free water or saline (Baxter Travenol Laboratories of Canada Ltd., Malton, Ontario), and all plastic test tubes and pipettes used were sterile. This minimized the possibility of contamination with endotoxin.

Measurement of Hemorrhage

Hemorrhage was quantitated with ^{59}Fe-labeled erythrocytes. In all experiments the erythrocytes were labeled *in vivo* in donor rabbits and transfused into the experimental animals according to the method of Kopaniak et al.[8]

The erythrocytes of donor rabbits were labeled by intravenous injections of ^{59}Fe-ferrous citrate (New England Nuclear, Lachine, Quebec) previously incubated with 5 ml of autologous serum (at 23 C for 10 minutes). Three to 4 days after labeling, anticoagulated (10 IU heparin/ml) blood containing 6×10^6 cpm of ^{59}Fe (on 500–2000 keV γ-spectrometer setting) was collected from the central ear artery of the donor rabbit. The ^{59}Fe-erythrocytes were lightly packed (200*g* for 10 minutes) and infused into the recipient rabbit's circulation 24 hours before sacrifice as described previously.[1] The amount of radioactivity per microliter of circulating blood in the recipient rabbit was determined, and this allowed conversion of the counts per minute of ^{59}Fe per lesion into an equivalent number of microliters of extravasated blood. ^{59}Fe was counted with the use of the γ-spectrometer window at 500–2000 keV, because this setting minimized the overlap of energy emission between ^{51}Cr and ^{59}Fe.

Morphologic Studies

Paraffin sections from tissue samples were prepared by first fixing the skin in 10% phosphate-buffered (pH 7.0) formaldehyde, dehydrating the sections, clearing them, and, following embedding, staining them with hematoxylin and eosin. For the preparation of semithin sections, 10 × 5 × 2-mm pieces of tissue were fixed in pH 7.4 phosphate-buffered glutaraldehyde (final concentration 2%) and formaldehyde (final concentration 10%) at 4 C overnight and dehydrated (without osmium postfixation) and embedded in hydroxyethyl methacrylate, and 1–2-μ sec-

tions were cut on a duPont Sorvall JB-4 microtome and stained with azure-eosin.[9]

Determination of Antibody Titer

It was found that approximately 70% of the population of rabbits obtained from the breeder had natural titers of circulating antibody (Ab) to *E coli*. The approximation of Ab titer involved the use of an agglutination test. Serial 1:2 dilutions of rabbit's serum in phosphate-buffered saline (PBS) (50 μl of each) were made in microtitration plates. To each dilution 50 μl of formaldehyde-killed and washed *E coli* (3×10^9/ml) were added, and the plates were incubated at 37 C for 60 minutes. The *E coli* suspension on glass slides from each well was then examined microscopically. Freely floating bacteria were taken to indicate lack of Ab in the serum, whereas agglutination of *E coli* was recorded as a positive titer. A number of wells were examined in order to estimate the titer of circulating Ab. Only rabbits with negative Ab titers were used *a*) in the controls (untreated), *b*) in the experiments with cobra venom factor (CoF), and *c*) in the experiments with leukocyte-depleted rabbits.

Hyperimmunization of Rabbits With *E Coli*

Rabbits were hyperimmunized with increasing doses of *E coli* injected intravenously. The animals were initially given injections twice every 10 days with 5×10^8–3×10^9 formaldehyde-killed *E coli*, and then live *E coli* (3×10^9 organisms) were injected several times at 10-day intervals. A total of 12 immunizing injections was made. Titers of induced Ab to *E coli* were estimated as described in the previous section.

Depletion of Hemolytic Complement

The hemolytic complement of rabbit serum was depleted with cobra venom factor (CoF) using a modified method of Cochrane et al[10] and Man and Minta.[11]

Naja naja kaouthia, Thailand cobra snake venom (Sigma Chemical Co., St. Louis, Mo), was fractionated by column chromatography (two steps) to obtain partially purified CoF. Two grams of the lyophilized cobra venom was dissolved in 100 ml of 0.01 M phosphate buffer (pH 7.5), and the sample was applied to the column. The conductivity of the applied sample was the same as the equilibrating buffer of the QAE-Sephadex. The QAE-Sephadex A-50 column was run at 4 C with an approximate flow rate of 20 ml/hr. Ten-milliliter fractions were collected (LKB Ultrarac fraction collector, LKB Produckter, Uppsala, Sweden). After application of the sample the column was washed with 0.01 M phosphate buffer, followed by a linear gradient of 0.01 M phosphate to 0.01 M phosphate with 0.4 M NaCl. A small volume of 0.01 M phosphate containing first 0.4 M NaCl and then 0.5 M NaCl followed the gradient. The collected fractions were tested for decomplementing activity, and the active ones were concentrated with positive pressure ultrafiltration with a UM-10 membrane (Amicon Corporation, Lexington, Mass). The concentrated sample was applied to a 2.5 × 100-cm Pharmacia glass column containing Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.01 M phosphate, 0.4 M NaCl buffer (pH 7.5). The column was run at 4 C, and the flow rate was adjusted to approximately 6 ml/hr. The protein elution profile was determined with the use of a Zeiss PMQII Spectrophotometer at 280 nm.

The decomplementing activity of the fractions was determined with the hemolytic assay of Mayer.[12] QAE fractions were tested by incubating 0.1 ml of the fraction material with 20 μl of normal human serum at 37 C for 30 minutes, with shaking. Following this incubation, 0.4 ml of Veronal buffered saline with Ca^{++}, Mg^{++} and 0.05% gelatin (VBS^{++}) and 0.5 ml (5×10^8/ml) of sheep red blood cells (EA) sensitized with hemolysin and suspended in VBS^{++} were added. Another incubation (60 minutes at 37 C, with shaking) followed and was stopped with 1.0 ml of cold veronal buffered saline with no metals, containing 0.01 M EDTA and 0.05% gelatin. The unlysed EAs were spun down (1400*g* for 10 minutes at 4 C). The amount of hemolysis in the supernatant was measured spectrophotometrically at the wavelength of 541 nm versus a control which contained no eluted material and thus measured spontaneous hemolysis. G-200 fractions were tested by the same method, but the volume of the G-200 eluate used was 50 μl. The active fractions (ie, those that had destroyed the hemolytic activity of normal human serum) were concentrated with the use of Amicon-positive pressure ultrafiltration and standardized according to the method of Cochrane et al.[10] The amount of CoF in 0.1 ml that reduced by 50% the hemolytic activity of a 1:20 dilution of normal human serum was defined as 1 unit. The CoF was then aliquoted and stored at −70 C until needed.

For decomplementation experiments *in vivo*, a total dose of 300 units of CoF per kilogram body weight of the rabbit was injected intravenously in four equal doses at 0, 4, 10, and 24 hours. In some experiments an additional injection of 250 units of CoF was made at 32–40 hours, and the rabbits were

used for intradermal lesions at 72 hours. The hemolytic activity of rabbit serum was determined before and during the experiment. The activity was measured by incubating 0.2 ml serial twofold dilutions of rabbit serum with 0.5 ml of EA (5×10^8/ml) in VBS^{++} for 60 minutes at 37 C and then stopping the reaction with 1.0 ml VBS-EDTA. The amount of hemolytic activity of rabbit serum was determined spectrophotometrically at 541 nm.

Leukocyte Depletion

Rabbit leukocytes were depleted with nitrogen mustard (Mustargen Hydrochloride, Merck Frosst Laboratories, Dorval, Quebec) according to a modified method of Thomas and Good.[13] The rabbits received 1.5 mg of nitrogen mustard per kilogram body weight as an intravenous injection. Benzathine Penicillin G (Penlong XL, Rogar/STB-BTI Products, London, Ontario) was injected subcutaneously (150,000 IU) in order to protect the animals against infection. Forty-eight hours later a second intravenous injection of 1.0 mg/kg nitrogen mustard was given. The experiments were performed 24 hours after the second injection of nitrogen mustard. The total leukocyte count per microliter of blood and a differential count were made prior to injection of nitrogen mustard and on the day of the experiment. The number of the remaining polymorphonuclear leukocytes per μl of blood was then determined.

Statistical Analysis

The Student *t* test for small samples was used to calculate *P* values.

Results

It was found, in agreement with previously published data,[1] that in *E coli*-induced lesions, hyperemia, infiltration of leukocytes, and vascular permeability occurred in the first 3–4 hours of lesion development and largely subsided by 6 hours, whereas hemorrhage usually continued to develop until 6 hours after the injection of bacteria and then remained at plateau levels (Figure 1). The changes in hyperemia, leukocyte accumulation, and vascular permeability were measured as rates, with the appropriate radioactive tracer injected in the final hour of the experiment. Hemorrhage, on the other hand, was quantitated as a cumulative parameter with ^{59}Fe-erythrocytes present in the circulation throughout the duration of the experiment.

The mediation of the inflammatory changes occurring in the *E coli* lesions was studied in different groups of rabbits. Since the host mediation systems were of primary interest, humoral factors of rabbits known to affect neutrophil function (*viz* the complement system and antibodies) were manipulated, and the development of *E coli* lesions in these rabbits was compared with normal control rabbits. In addition to the hyperimmunization of the rabbits with *E coli* and partial depletion of the hemolytic complement, the rabbits were also depleted of leukocytes.

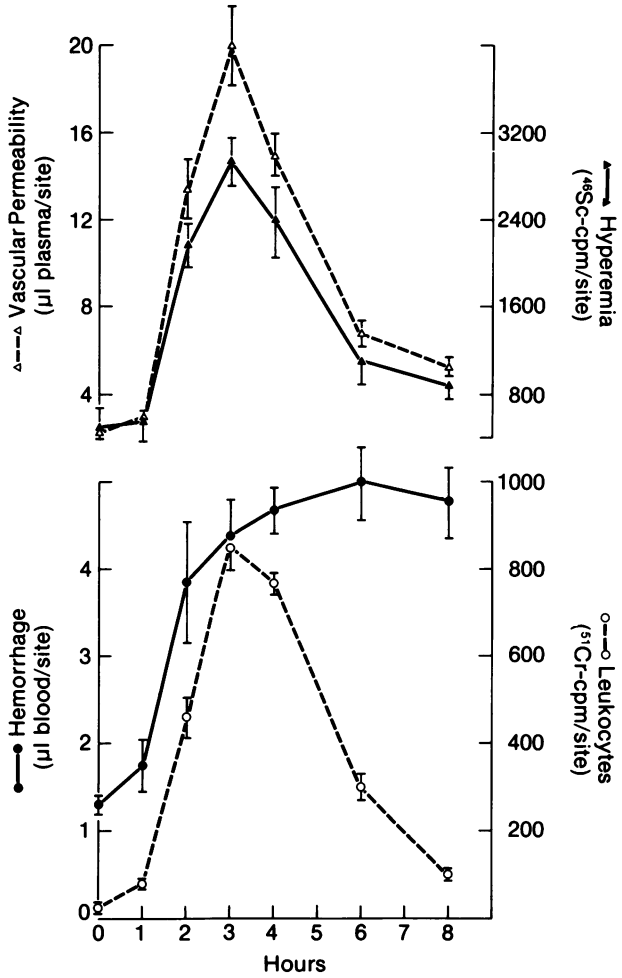


Figure 1—Kinetics of the acute inflammatory reaction to intradermal injections of *E coli* in a normal rabbit. Four parameters of the inflammatory response were measured with radioactive tracers. The figure is representative of an experiment performed in one animal with data points representing the mean ± standard error of five replicate sites injected at each time point. The hours show the age of the lesion at the time the rabbit was sacrificed. Control skin sites are represented as the point at time 0 hours. Hyperemia and leukocyte accumulation are represented as counts per minute of their appropriate isotopes, while vascular permeability and hemorrhage are expressed as microliters of extravasated plasma and blood, respectively. In this experiment hyperemia, enhanced vascular permeability, and leukocyte accumulation (all of which were measured as rates) reached maximal values in 3-hour-old lesions and subsided by 6 hours. Hemorrhage, which was measured as a cumulative parameter, increased rapidly up to 3 hours and was followed by a less steep rise up to 6 hours. For further details see Tables 1-5.

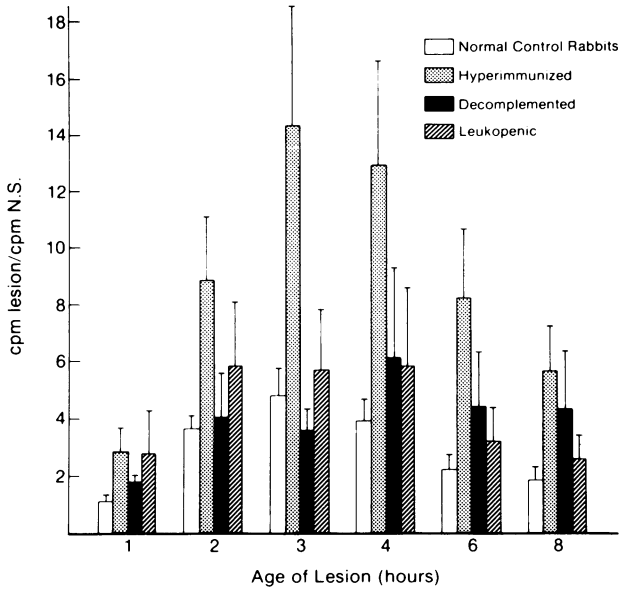


Figure 2—Hyperemia in skin lesions induced with *E coli* in four different groups of rabbits. *Open bars* represent normal rabbit controls, *dotted bars* the hyperimmunized group of animals, *solid bars* the decomplemented rabbits, whereas *cross-hatched* bars depict leukopenic animals. Hyperemia is expressed as fold increase in blood flow over control skin—ie, as a ratio of the mean of the microsphere-associated radioactivity of six replicate lesions at each injection time to the mean of six control skin sites obtained for each rabbit. The *bars* represent the mean ± standard error of these ratios obtained from several rabbits in each group, at each injection time. The data are so summarized for reasons of clarity and for observation of trends in kinetics and extent of hyperemia in the different groups of rabbits. Maximal hyperemia was reached between 2 and 4 hours in the different groups, and no significant differences in the extent of hyperemia were observed between normal, decomplemented, and leukopenic animals. The hyperimmunized group showed greater hyperemia than normal rabbits, and the difference was significant at each time point ($P < 0.01$).

The rabbits used as normal controls were selected as having no natural antibody to *E coli*. In the hyperimmunized group of rabbits the titers of antibody in serum ranged between 1:4096 and 1:4194304 (ie, 2^{-12} and 2^{-22}), but most were around 1:1048576 (2^{-20}). No difference in the extent of response to *E coli* was found in rabbits with different titers, probably because of the availability of excess antibody. The complement-depleted animals were used only when the titer of hemolytic complement at the time of the experiment was 10% or less than before treatment with CoF. No attempt was made to measure the individual components of the complement system. The normal number of white blood cells per microliter of rabbit blood is 5000-13,000, of which 30-50% are neutrophils. In nitrogen-mustard-treated rabbits the circulating neutrophils were 2400-3800/μl before treatment and were depleted to 0-60 polymorphonuclear leukocytes (PMNs)/μl of blood after drug treatment.

Although conditions were kept constant within a group of rabbits, the rabbits exhibited some biologic variability in the response, making impossible the use of mere counts per minute as an expression of the results. This was the reason for a normalizing factor, using an internal control for each rabbit, which would allow the data within a group to be combined and the trends and differences in the extent of the response between groups to be compared. Thus, large numbers of data were summarized for clarity. *E coli* lesions for each time point were reproduced five or six times in one animal. Thus, for six different time points and control sites, there were 35 to 42 lesions in a rabbit. (Pilot experiments were performed to ascertain that deposition of the early lesions and a large number of these sites did not affect the development of the inflammatory reaction at any time point.) For each time point within one rabbit the mean ± standard error (SEM) of the counts per minute for each parameter was calculated. The normalizing factor was used as follows. As described in Materials and Methods for hyperemia, leukocyte accumulation and vascular permeability the fold increase of the response over control skin sites was calculated:

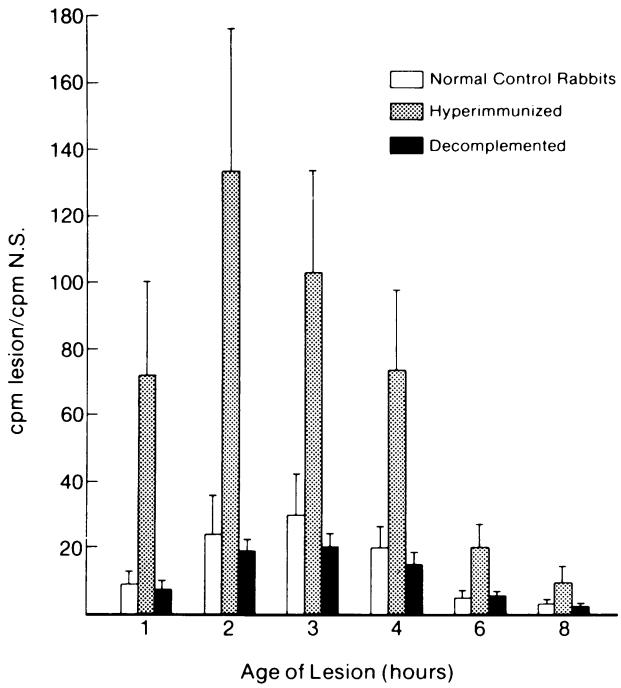


Figure 3—Accumulation of ^{51}Cr-labeled leukocytes in *E coli* lesions of different ages in three groups of rabbits is represented. The leukocyte accumulation in the lesions is expressed as a ratio of ^{51}Cr-associated radioactivity in lesions to the radioactivity in control skin sites. The *bars* represent the mean ± standard error of data obtained from individual rabbits (as explained in Figure 2) and combined to give a "group average." The accumulation of ^{51}Cr-leukocytes was greater in hyperimmunized animals than in normal ones, with $P < 0.05$ (df = 8) at 2 hours. There was no significant difference in leukocyte accumulation between the normal and the decomplemented group. The maximal leukocyte infiltration occurred earlier (at 2.2 ± 0.2 hours) in hyperimmunized rabbits than it did in normal rabbits (3.2 ± 0.2 hours). The difference in the time of peak response is significant ($P < 0.01$).

$$\frac{\text{cpm/lesion}}{\text{cpm/control site}} = \text{fold increase}$$

The mean increases from all the rabbits within a group were combined for each time point, and the mean ± standard error of the mean (SEM) was calculated. The bars in Figures 2, 3, and 4 represent such means of the means ± SEM. In more recent experiments, vascular permeability in the lesions was expressed as equivalents of extravasated plasma calculated by use of the formula:

$$\frac{^{125}\text{I cpm/lesion}}{^{125}\text{I cpm/}\mu\text{l plasma}}$$

Hemorrhage was expressed as microliter equivalents of extravasated blood, calculated by dividing ^{59}Fe counts per minute per lesion by ^{59}Fe counts per minute per microliter of circulating blood.

Table 1 is based on recent studies in which plasma levels for ^{125}I-albumin were available and shows the numeric data expressing vascular permeability in microliters of extravasated plasma and as fold increases over control skin sites. The data are based on 7 normal rabbits and are derived from the same original counts per minute of ^{125}I per lesion in both columns. The only difference between the left and right columns is the units in which increased vascular permeability is expressed. Skin sites in which normal saline was injected or control skin sites showed 2.82 ± 0.63-μl equivalents of extravascular plasma (following massaging out of larger blood vessels—see Materials and Methods). At 2 hours 15.77 ± 5.17 μl of plasma (ie, approximately 5 times NS levels) were found in *E coli* lesions.

Table 1—Vascular Permeability in Intradermal Lesions Induced With *E coli* in 7 Normal Rabbits

Hours*	Mean ± SEM (% SEM) (μl plasma)	Mean ± SEM (% SEM) (cpm lesion/cpm NS)
1	6.22 ± 1.49 (24)	2.25 ± 0.29 (13)
2	15.77 ± 5.17 (33)	4.95 ± 0.99 (20)
3	14.85 ± 2.70 (18)	4.92 ± 0.97 (20)
4	11.43 ± 1.39 (12)	3.93 ± 0.96 (24)
6	6.24 ± 0.60 (10)	2.32 ± 0.48 (22)
8	6.52 ± 0.83 (13)	2.51 ± 0.56 (22)
NS†	2.82 ± 0.63 (22)	

* Age of lesion in hours.
† Normal skin control and normal saline control.
All procedures are described in Materials and Methods.

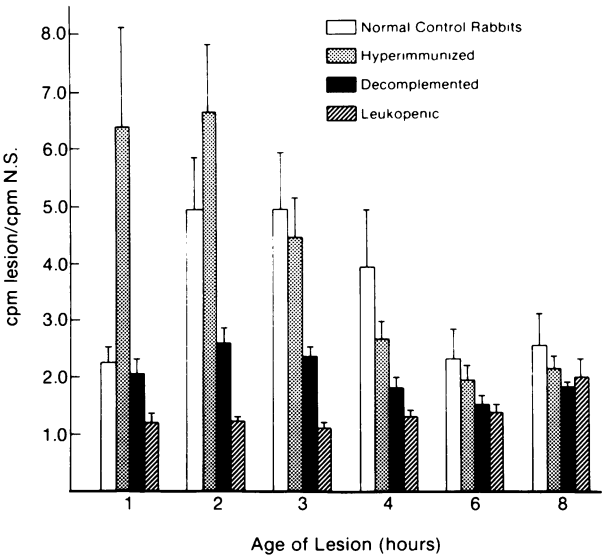


Figure 4—Enhancement in vascular permeability in intradermal lesions induced with *E coli* is compared between the four groups of rabbits. The vascular permeability is expressed as fold increase over control skin sites obtained for each experimental animal, as explained in Figure 2. The *bars* represent the mean ± standard error of values obtained from several rabbits within one group. Vascular permeability in hyperimmunized rabbits was greater at 1 hour than in normal controls, but no significant difference between these two groups was observed at later times. The decomplemented animals' vascular permeability was significantly ($P < 0.05$ to $P < 0.02$, df = 13) lower than that of normal control rabbits between 2 and 4 hours. Leukopenic animals showed almost no increase in vascular permeability (lesion to control skin ratio ~1) throughout the experiment, being significantly different at 1-4 hours from normal.

Hyperemia was measured in the four groups (Fig. 2), and in all groups the maximal response occurred between 2 and 4 hours. Depletion of hemolytic complement to less than 10% of the original serum levels or depletion of leukocytes with nitrogen mustard did not affect the kinetics or the extent of the hyperemia, in comparison with normal rabbits. In all these groups maximal hyperemia reached 4–6 times the blood flow of control skin. The hyperimmunized group of rabbits showed greater hyperemia than normal controls, ie, approximately a 14-fold increase over control skin. The difference was significant ($P < 0.01$). Table 2 shows the numeric values on which Figure 2 is based.

Leukocyte accumulation in normal, hyperimmunized, and decomplemented rabbits was measured (Figure 3). The accumulation of ^{51}Cr-leukocytes was greater in hyperimmunized animals (133-fold increase over control skin) than in normal controls (24-fold increase). The difference was significant ($P < 0.05$ at 2 hours). The maximal leukocyte infiltration occurred earlier (2.2 ± 0.2 hours) in the hyperimmunized group than in normal rabbits (3.2 ± 0.2 hours). The difference in the time of peak response was significant ($P < 0.01$). Decomplemented rabbits showed no significant difference in either the kinetics or the magnitude of leukocyte accumulation from normal animals (Table 3).

Figure 4 represents the values of enhanced vascular permeability as measured in the four experimental groups of animals. Maximum protein leakage in 2-and 3-hour-old lesions in normal rabbits reached fivefold increases over control skin sites. The only significantly different ($P < 0.05$) permeability be-

Table 2—Hyperemia in Intradermal Lesions Induced With *E coli* in Rabbits

Hours*	Normal rabbits (n = 6)†	Hyperimmunized rabbits (n = 5)†	Decomplemented rabbits (n = 3)†	Leukopenic rabbits (n = 6)†
1	1.22 ± 0.22 (18)	2.90 ± 0.81 (28)	1.84 ± 0.23 (13)	2.85 ± 1.47 (51)
2	3.70 ± 0.52 (14)	8.96 ± 2.19 (24)	4.11 ± 1.58 (38)	5.90 ± 2.33 (39)
3	4.90 ± 1.00 (20)	14.40 ± 4.12 (29)	3.69 ± 0.72 (20)	5.83 ± 1.96 (34)
4	4.03 ± 0.88 (22)	12.94 ± 3.73 (29)	6.21 ± 3.17 (51)	5.97 ± 2.69 (45)
6	2.27 ± 0.52 (23)	8.28 ± 2.39 (29)	4.40 ± 1.85 (42)	3.29 ± 1.10 (33)
8	1.99 ± 0.45 (22)	5.77 ± 1.58 (27)	4.35 ± 2.17 (50)	2.59 ± 0.84 (32)

* Age of lesion in hours.
† Mean ± SEM (% SEM), cpm lesion/cpm normal skin control and normal saline control.
All procedures are described in Materials and Methods.

tween normal and hyperimmunized groups occurred in 1-hour-old lesions, where hyperimmunized rabbits showed a sixfold increase versus a twofold increase in normal rabbits (Figure 4 and Table 4). The vascular hyperpermeability of decomplemented animals was significantly lower (twofold increase) than that of normal control rabbits ($P < 0.05$ to $P < 0.02$) between 2 and 4 hours. Leukopenic animals showed almost no increase in vascular permeability throughout the experiment. The lesion to control skin ratio was close to unity in all *E coli* sites, except the 8-hour-old ones, indicating that no enhancement in protein leakage occurred. The difference between leukopenic and normal rabbits was significant at 1–4 hours ($P < 0.10$ to $P < 0.02$).

No significant difference was found in the amount of hemorrhage between the normal, hyperimmunized, and decomplemented groups of rabbits (Figure 5). All these groups showed between 1 μl of blood at 1 hour and 17 μl in 8-hour-old lesions (Table 5). The amount of erythrocytic extravasation in leukopenic rabbits, on the other hand, was significantly lower than in normal controls, reaching 4.67 ± 1.52 (SEM) μl of blood in 8-hour-old lesions (P values ranged from $P < 0.001$ at 3 hours to $P < 0.1$ at 6 hours. The 8-hour-old lesion were not significantly different from the normal lesions). The amount of blood in control skin sites was the same in all groups (1.30–1.52 μl).

Histologic examination of the lesions of decomplemented rabbits did not reveal any qualitative or quantitative differences from normal rabbits. There were more numerous PMNs in the hyperimmunized than in the normal rabbits, and this difference was more definite in the older (4–8 hour) lesions (Figures 6 and 7). However, in the leukopenic animals, very definite differences in the appearance of the *E coli* lesions were observed when their injection sites were compared with those of normal animals. In the normal and hyperimmunized rabbits PMN infiltration was first noticed as focal accumulations, usually around vessels in 1-hour-old lesions, and did not increase in number beyond 6-hours. By 3 hours a small number of rod-shaped bacteria were still recognizable with an oil immersion objective (Figure 8). On the other hand, innumerable rod-shaped bacteria were lying free in the dermis of leukopenic rabbits (Figure 9). In 6-hour-old lesions no gross signs of inflammation were visible in the leukocyte-depleted animals, whereas in the normal and hyperimmunized rabbits there was pronounced swelling and hemorrhage. At low magnification a very intense infiltration of the dermis had taken place, as described above (Figures 6 and 7), but in nitrogen-mustard-treated rabbits the field seemed normal at low magnification, except that there were still clumps of bacteria between the collagen bundles of the dermis (Figure 10). At high magnification in 6-hour-old injection sites of neutrophil-depleted rabbits some of the accumulations of bacteria contained occasional PMNs and monocyte/macrophages (Figure 11).

Table 3—Leukocyte Infiltration in Intradermal Lesions Induced With *E coli* in Rabbits

Hours*	Normal rabbits (n = 5)†	Hyperimmunized rabbits (n = 5)†	Decomplemented rabbits (n = 6)†
1	9.03 ± 4.63 (51)	72.26 ± 26.84 (37)	8.29 ± 1.97 (24)
2	24.27 ± 11.8 (49)	133.58 ± 43.09 (32)	19.04 ± 3.79 (20)
3	30.41 ± 12.5 (41)	103.62 ± 30.50 (29)	20.33 ± 3.95 (19)
4	20.51 ± 6.52 (32)	73.36 ± 24.48 (33)	15.45 ± 3.28 (21)
6	4.90 ± 1.93 (39)	19.66 ± 6.88 (35)	5.29 ± 1.15 (22)
8	2.78 ± 0.66 (24)	9.34 ± 4.98 (53)	3.17 ± 0.69 (22)

* Age of lesion in hours.
† Mean ± SEM (% SEM), cpm lesion/cpm normal skin control and normal saline control.
All procedures are described in Materials and Methods.

Table 4—Vascular Permeability in Intradermal Lesions Induced With *E coli* in Rabbits

Hours*	Normal rabbits (n = 7)†	Hyperimmunized rabbits (n = 6)†	Decomplemented rabbits (n = 8)†	Leukopenic rabbits (n = 4)†
1	2.25 ± 0.29 (13)	6.40 ± 1.76 (27)	2.04 ± 0.25 (12)	1.20 ± 0.12 (10)
2	4.95 ± 0.99 (20)	6.64 ± 1.15 (17)	2.61 ± 0.29 (11)	1.24 ± 0.06 (5)
3	4.92 ± 0.97 (20)	4.45 ± 0.70 (16)	2.35 ± 0.16 (7)	1.16 ± 0.07 (6)
4	3.93 ± 0.96 (24)	2.67 ± 0.31 (12)	1.80 ± 0.20 (11)	1.31 ± 0.11 (8)
6	2.32 ± 0.48 (21)	1.94 ± 0.22 (11)	1.52 ± 0.13 (9)	1.41 ± 0.11 (8)
8	2.51 ± 0.56 (22)	2.15 ± 0.26 (12)	1.81 ± 0.08 (5)	2.00 ± 0.32 (16)

* Age of lesion in hours.
† Mean ± SEM (% SEM), cpm lesion/cpm normal skin control and normal saline control.
All procedures are described in Materials and Methods.

Discussion

The experiments reported in this paper were performed in order to elucidate the mediation systems of the acute inflammatory response to *E coli*. It was observed in the course of the study that certain parameters of the inflammatory reaction worked "in concert" and seemed to be affected one by the other. In particular, it was noted that manipulation of the host leukocytes resulted in pronounced changes in vascular permeability and hemorrhage development in *E coli*-induced inflammatory lesions. Modifications in the host mediation systems were then performed to check the premise that rabbit leukocytes, predominantly polymorphonuclear, mediate vascular injury, manifested as an increase in vascular permeability and hemorrhage.

The main events in the acute inflammatory reaction are changes in local blood flow, exudation of plasma due to an increase in vascular permeability and accumulation of neutrophils, and, in fewer numbers, monocytes in the inflammatory lesion. In more severe injury, such as found with the *E coli* organisms, extravasation of erythrocytes also occurs, resulting in

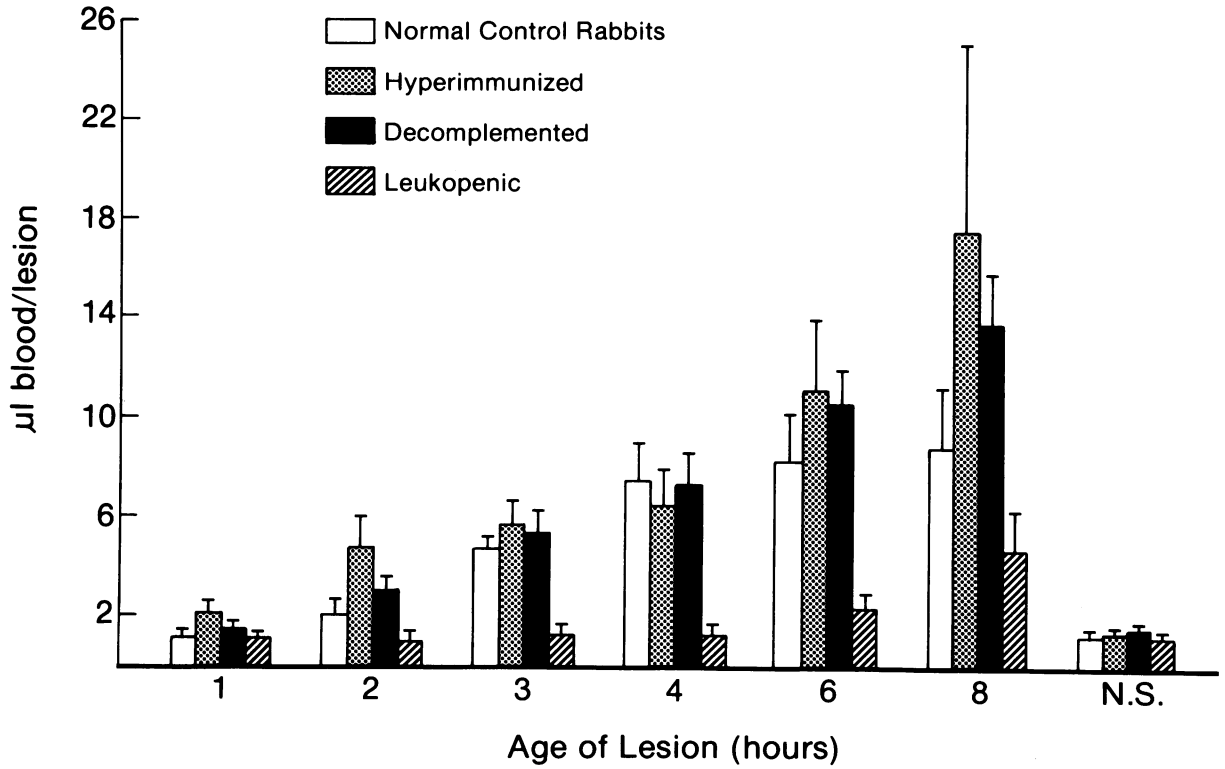


Figure 5—Development of hemorrhage in four groups of rabbits given intradermal injections of *E coli* is shown. Hemorrhage is expressed as microliter equivalents of blood per lesion. The *bars* represent the mean ± standard error of values obtained from several rabbits within a group. No difference was observed in the extent of hemorrhage between normal, hyperimmunized, and decomplemented groups of rabbits. The amount of erythrocyte extravasation in leukopenic rabbits was significantly lower than in normal rabbits ($P < 0.001$ at 3 hours and $P < 0.01$ at 6 hours with 5 df). There was no difference between groups in the amount of blood contained in control skin sites (NS).

Table 5—Hemorrhage in Intradermal Lesions Induced With *E coli* in Rabbits

Hours*	Normal rabbits (n = 4)†	Hyperimmunized rabbits (n = 6)†	Decomplemented rabbits (n = 9)†	Leukopenic rabbits (n = 3)†
1	1.19 ± 0.27 (23)	2.06 ± 0.51 (25)	1.44 ± 0.21 (14)	1.17 ± 0.30 (26)
2	2.09 ± 0.61 (29)	4.75 ± 1.11 (23)	3.04 ± 0.45 (15)	1.09 ± 0.26 (24)
3	4.79 ± 0.11 (2)	5.67 ± 1.04 (18)	5.48 ± 0.93 (17)	1.31 ± 0.38 (29)
4	7.55 ± 1.68 (22)	6.41 ± 1.49 (22)	7.30 ± 1.23 (17)	1.43 ± 0.31 (21)
6	8.28 ± 1.90 (23)	11.03 ± 3.00 (27)	10.85 ± 1.68 (16)	2.51 ± 0.35 (14)
8	8.86 ± 2.44 (27)	17.45 ± 7.57 (43)	13.90 ± 2.06 (15)	4.67 ± 1.52 (33)
NS‡	1.35 ± 0.31 (23)	1.37 ± 0.31 (23)	1.52 ± 0.20 (13)	1.30 ± 0.08 (6)

* Age of lesion in hours.
† Mean ± SEM (% SEM) in microliters of blood.
‡ Normal skin control and normal saline control.
All procedures are described in Materials and Methods.

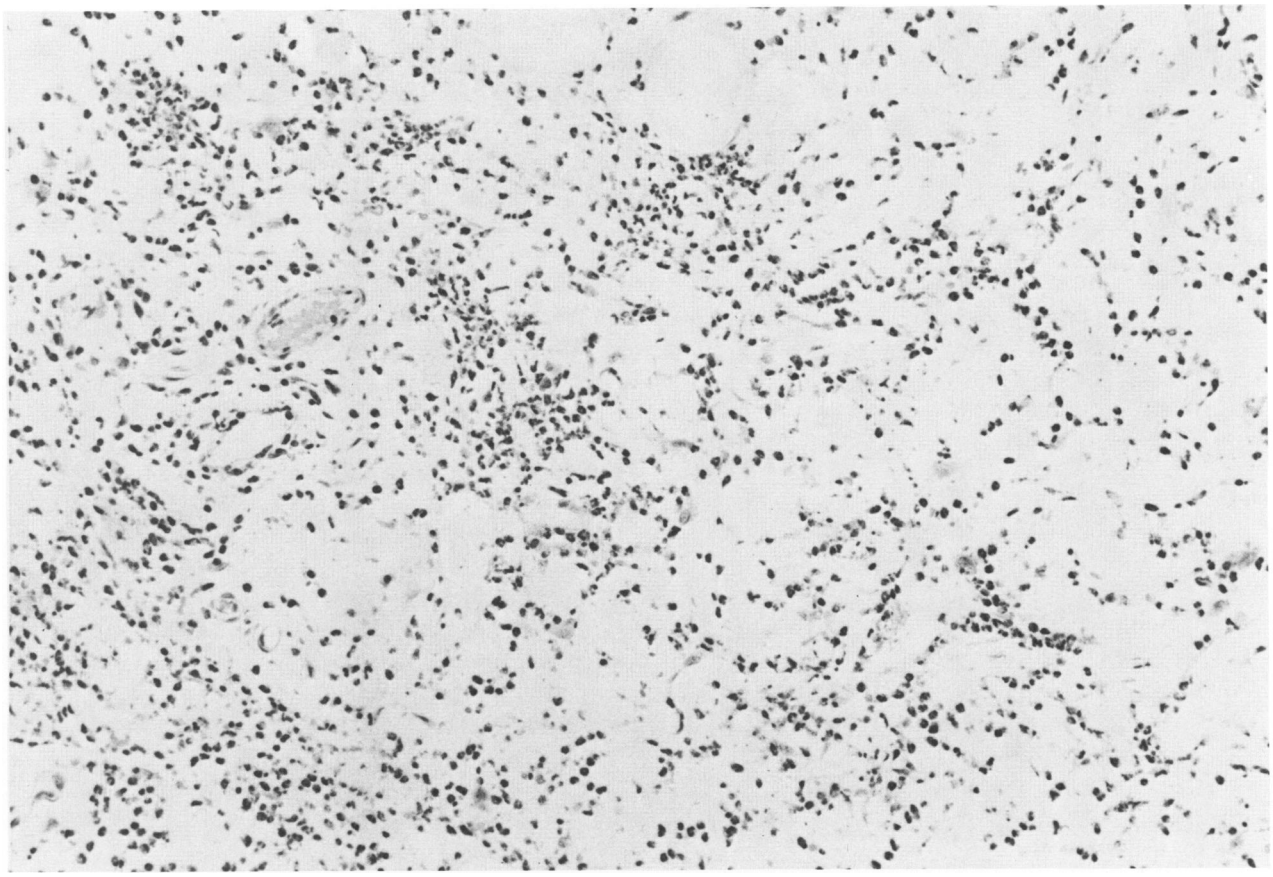
local hemorrhage. All these changes were quantitated with the aid of radioactive tracers, appropriate for each of the parameters measured.

The studies described in this paper indicate that in the inflammatory lesions induced in rabbits with *E coli* hyperemia, enhanced vascular permeability and maximal leukocyte accumulation occur almost simultaneously. These lesions develop early in the inflammatory reaction, becoming measurable (and, in the case of neutrophils, histologically apparent) within the first hour after the injection of bacteria. Of these changes, hyperemia and enhanced vascular permeability are transient, affecting the microvasculature for several hours without, however, causing extensive vascular damage, ie, disruption of the vascular wall and grossly visible hemorrhage. Accumulation of leukocytes is associated with engulfment of the bacteria and degranulation.[1] The simultaneously occurring increase in vascular permeability seems to depend primarily on PMN-derived mediators, eg, cationic proteins, proteolytic enzymes. Hemorrhage, on the other hand, reaches a peak after the leukocytes have emigrated and, in fact, at a time when the emigrated PMNs have reached the end of their life span. At least in the blood the half-life of a rabbit PMN is about 3.2–3.8 hours.[7] The discrepancy between the early increase in vessel permeability and leukocyte accumulation and late hemorrhage is even more evident in lesions induced with C5a des Arg.[14,15] There is good reason to believe that the hemorrhage is due to a massive release of lysosomal proteases, but no definitive proof is available for this assumption as yet.

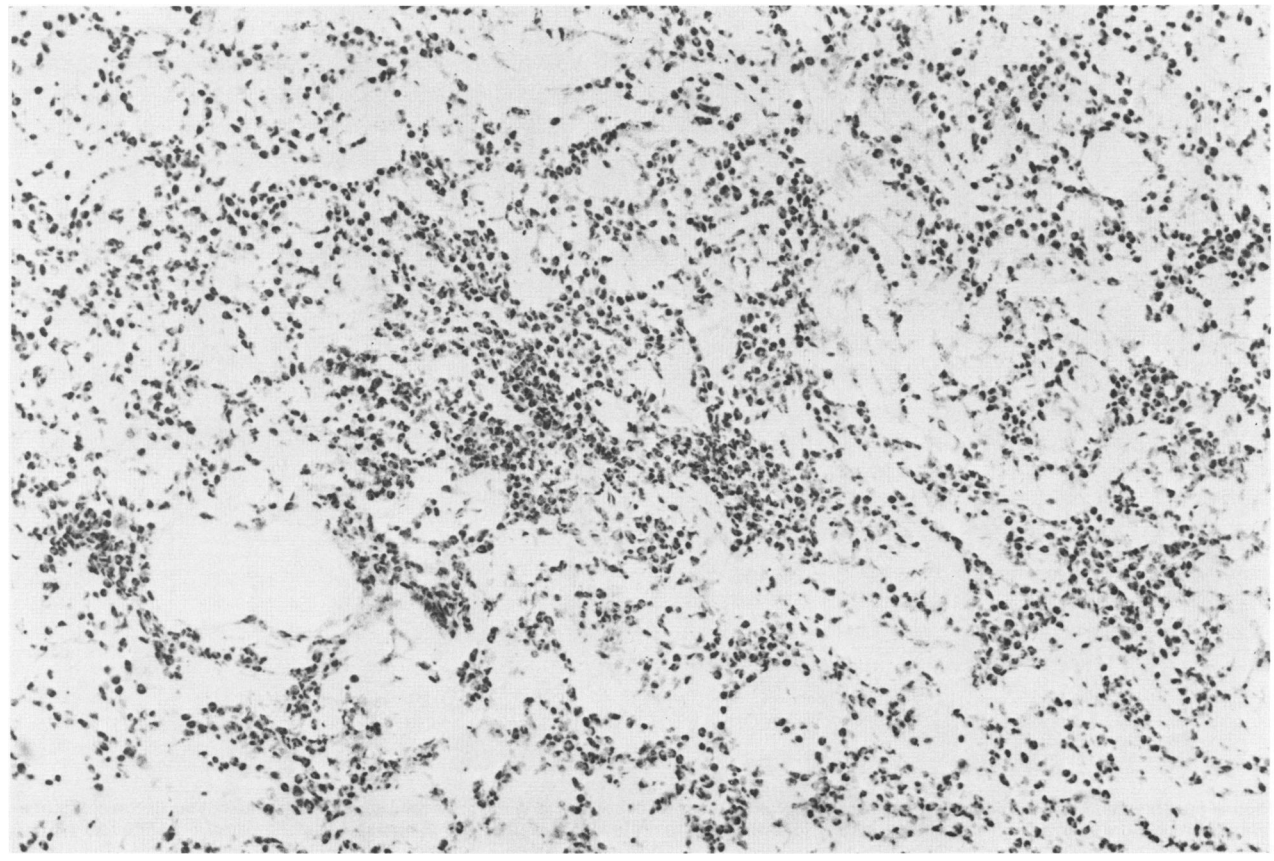
Hyperemia in *E coli* lesions was not affected by depletion of hemolytic complement nor by leukopenia induced with nitrogen mustard. Hyperimmunization of the animals, on the other hand, caused a 200% increase in hyperemia over values found in normal rabbits. The mediation of hyperemia in the hyperimmunized rabbits is not understood. Studies with *E coli* opsonized *in vitro* injected intradermally into one-half of the back of rabbits did not produce greater hyperemia values than nonopsonized *E coli* lesions deposited into the second half of the backs of the rabbits (unpublished data). It is possible that *in vitro* opsonization does not reflect all the processes occurring in the hyperimmunized rabbits. The role of hyperimmunization implies greater availability of antibody and hence complement, acting to attract more PMNs into the site, and yet the increase in blood flow is not neutrophil-mediated, since this parameter was not decreased in leukopenic rabbits. The best candidates for mediation of hyperemia are the vasodilatory prostaglandins.[4] Johnson et al[16] detected PGE_2 and $PGF_{2\alpha}$ in the lymph draining *E coli*-induced lesions. The levels of PGE_2 and $PGF_{2\alpha}$ rose early in the inflammatory response, reaching maximal values in the first 10 hours and then subsiding. Preliminary studies show that the inflammatory reaction induced in the rabbit with *E coli* can be partially inhibited by such inhibitors of prostaglandin cycloxygenase as ASA or indomethacin.[17]

Accumulation of leukocytes in intradermal *E coli* lesions was not significantly decreased in animals depleted of complement with cobra venom factor. The depletion of hemolytic complement is achieved by intravenous (or intraperitoneal) injections of CoF, which circulates in plasma, with a half-life of 32 hours.[10] It is possible that CoF did not deplete C5 levels, and this was not tested; merely CH_{50} titration was performed. There exists a possibility that complement is present in the circulation in such an excess that depletion to 10% of the original levels still leaves enough to mediate the chemotaxis of PMNs. It is also possible that interstitial cells such as macrophages

Figure 6—Infiltration of the dermis by leukocytes in a 6-hour-old inflammatory lesion elicited in a normal rabbit by injection of formalin-killed *E coli*. At high magnification over 95% of the infiltrating cells were PMN leukocytes (compare with Figures 7 and 10). (Methacrylate-embedded tissue, 1-μ thick section, azure-eosin, × 188) **Figure 7**—Lesion similar to the one in Figure 6, but elicited in a hyperimmunized rabbit (compare with Figures 6 and 10). (× 188)

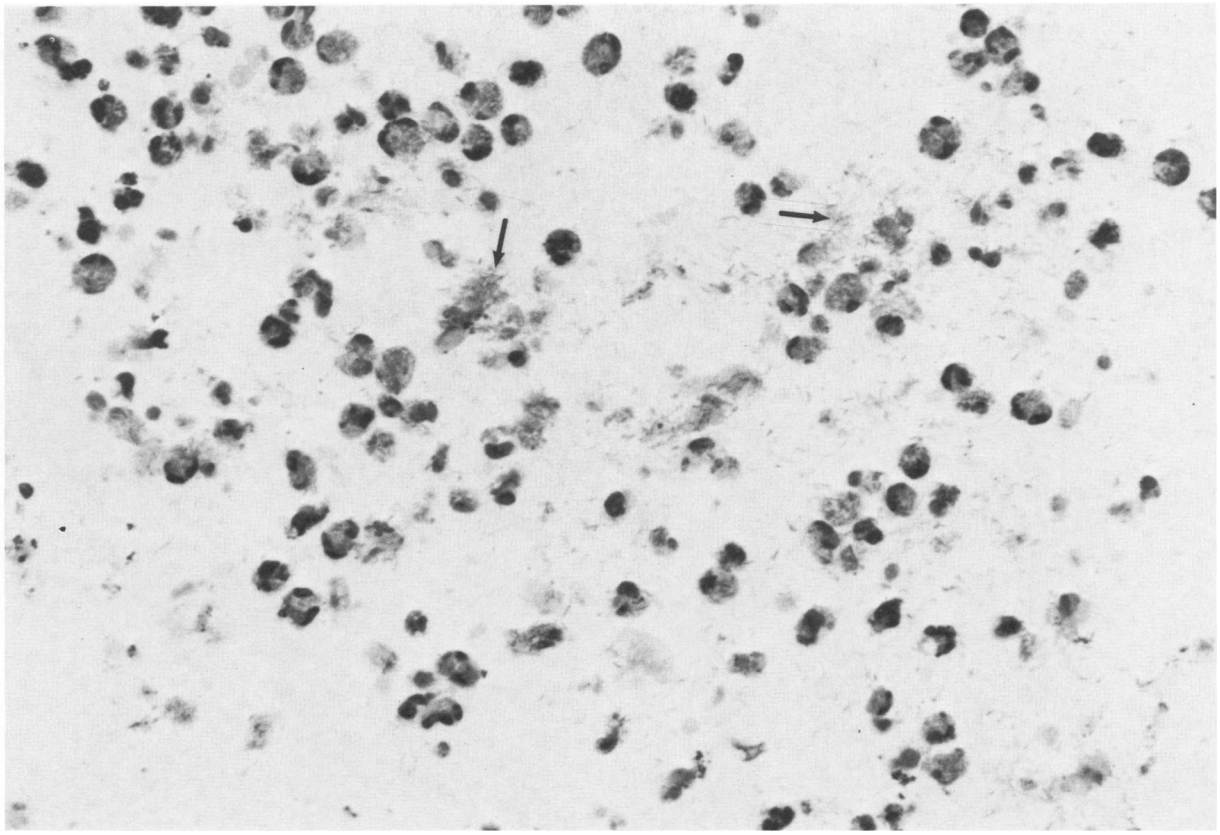


6



7

8



9

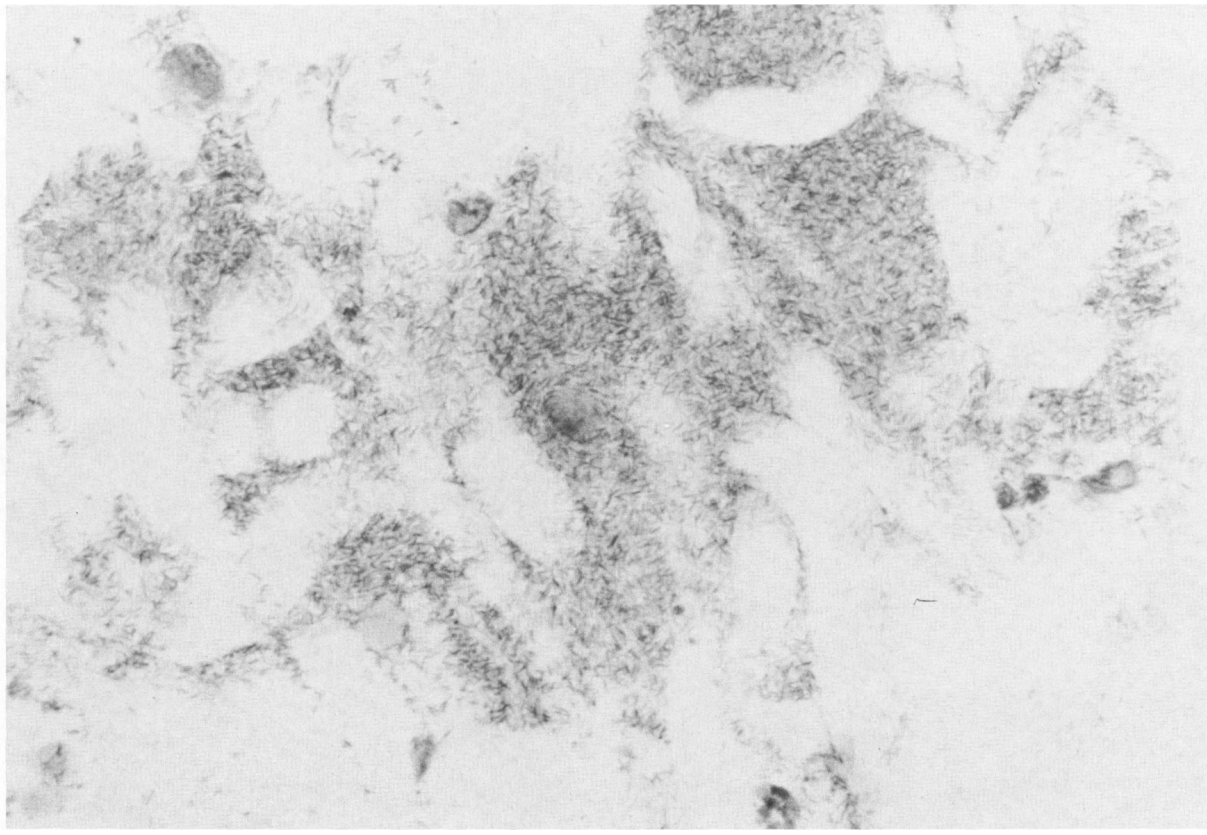
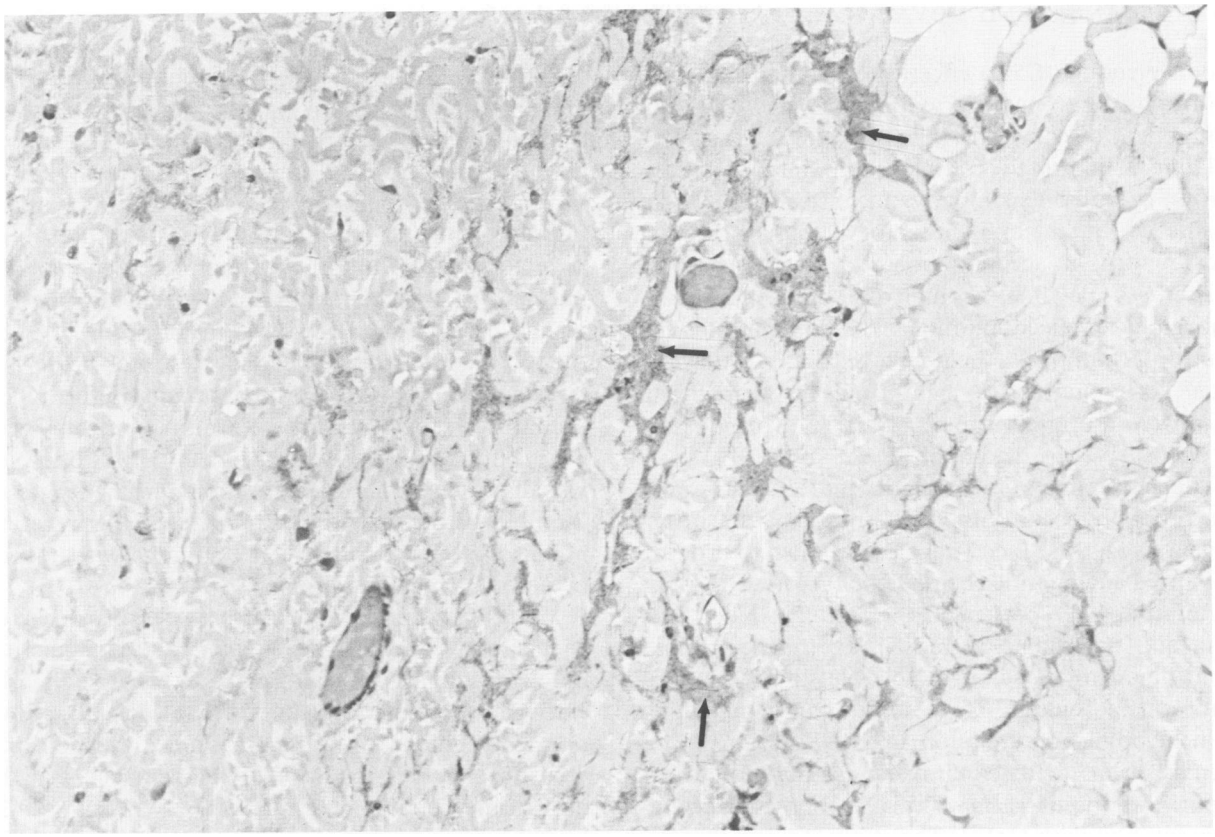
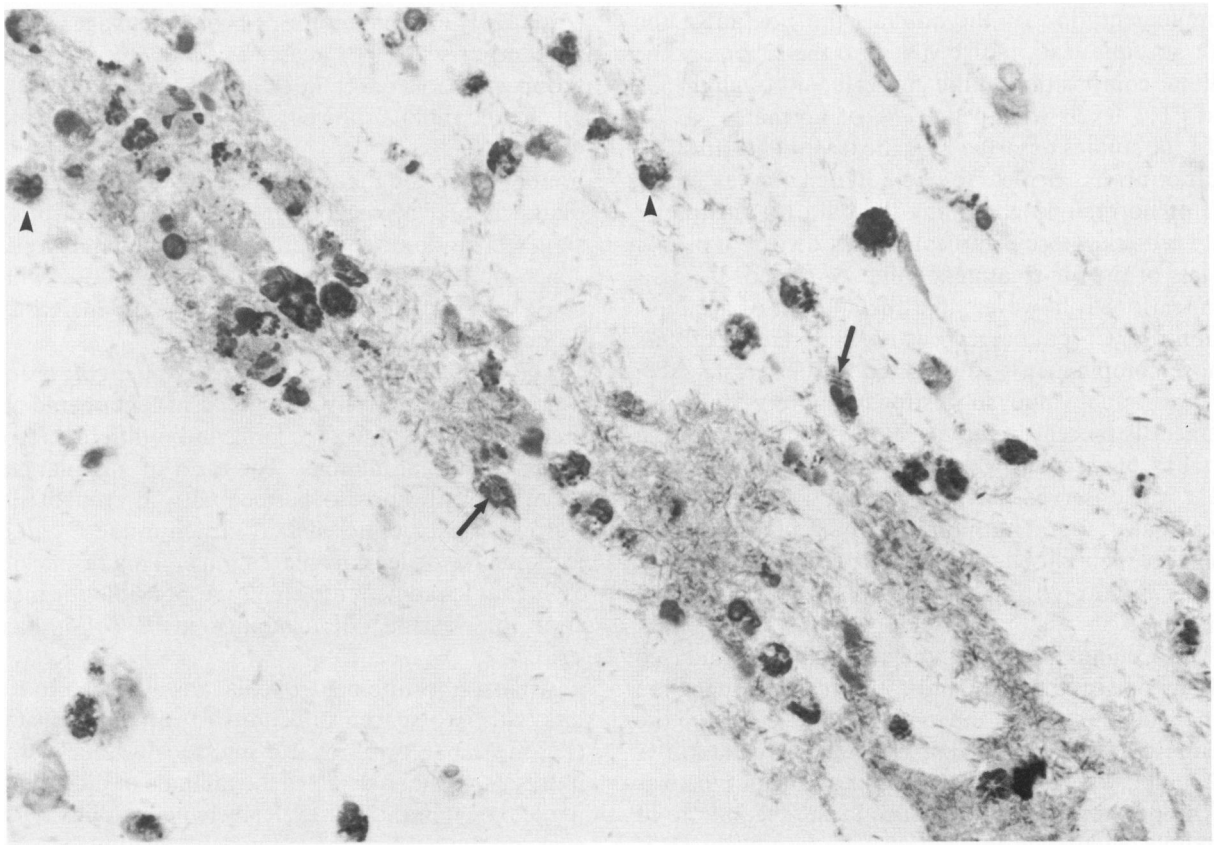


Figure 8—Three-hour-old lesion induced in a normal rabbit. Free rod-shaped bacteria are seen between the PMN leukocytes (*arrows*). No free-lying bacteria were seen at high magnification in lesions such as illustrated in Figures 6 and 7 (6 hours). Tissue prepared as in Figure 6. (× 752)
Figure 9—Skin site of a leukopenic rabbit given an injection 3 hours previously of *E coli*. Numerous free bacteria lie in the dermis, which is not infiltrated by leukocytes. (Tissue prepared as in Figure 6)



10



11

Figure 10—Skin site of a leukopenic rabbit given an injection 6 hours previously of bacteria. Note the clumps of bacteria (*arrows*) between bundles of collagen (compare with Figures 6 and 7). (Tissue prepared as in Figure 6, but stained with Azure II) **Figure 11**—Skin site of a leukopenic rabbit given an injection 6 hours previously of *E coli*. There are small accumulations of PMN leukocytes (*arrows*), monocytes (*arrowheads*), and numerous free-lying bacteria. (Tissue prepared as in Figure 6, × 752)

produce enough C5 to effect the chemoattraction of neutrophils into intradermal lesions and that this component of complement is inaccessible to the circulating CoF–protein complex. Pulmonary administration of immune complexes into C5-deficient mice resulted in either no inflammatory response or mild, focal neutrophil accumulation.[18]

At the same time, normal, C5-sufficient controls showed generalized pulmonary inflammation with neutrophil infiltrates, hemorrhage, and edema. The focal accumulations of neutrophils in C5-deficient mice were proposed to occur because of production of C5 by alveolar macrophages.

The attraction of neutrophils into *E coli* lesions can probably occur without the participation of endogenous factors. *E coli* have been shown to produce both lipid and peptide chemotactic factors.[19-21] These factors might be present in the formalin-killed bacteria and participate in mobilizing the neutrophils from blood to tissue. It is possible, therefore, that complement does not play a unique role in mediation of neutrophil accumulation in *E coli* lesions. This premise seems to be confirmed by a study of pyelonephritis induced in rats with *E coli* following decomplementation with CoF.[22] The study showed that decomplementation of the animals did not affect the rate of sequestration of PMNs into the lesions or the cellular composition of the infiltrate, although the C3 and CH_{50} levels were below 2% of normal.

In the studies reported here the treatment with CoF did not always deplete the hemolytic complement of 2% of normal. In compiling the data for this paper only rabbits whose complement was depleted to 10% or less of the pre-treatment value were used. It might be mentioned, however, that the amount of accumulation of ^{51}Cr-leukocytes was not different in rabbits whose complement was depleted to 2% versus 50% of the original value, suggesting once more that complement is present in large excess. An increase in the number of circulating neutrophils after CoF treatment was observed in this study, as well as by other investigators.[10,22] The numbers of circulating PMNs vary between rabbits, and this parameter was not closely monitored in the decomplemented group of animals.

The accumulation of leukocytes in hyperimmunized rabbits occurred earlier and to a greater extent than in normal animals. In the hyperimmunized group the mean time ($\bar{t}$) of maximal response occurred at 2.2 ± 0.2 hours, whereas in normal animals mean time was 3.2 ± 0.2 hours. The difference in the mean time of maximal leukocytic infiltration was significant ($P <$ 0.01). This earlier induration and increase in density was observed grossly by palpation and by transillumination respectively and later confirmed by isotope counting and histologic examination. It should be mentioned that comparisons of the extent of response between groups of rabbits, to be meaningful, should be made at the time of maximal occurrence of the parameter (for accumulation of leukocytes, between 1 and 4 hours). At 2 hours after the injection of *E coli* the rate of infiltration of leukocytes into the dermal lesions is approximately 5 times greater in the hyperimmunized group than in the normal control group. This difference is significant ($P < 0.05$). As observed visually, the amount of induration and the length of duration of the reaction was greater in the hyperimmunized group of animals, as compared with normal or decomplemented rabbits.

Vascular permeability in *E coli* lesions occurred slightly earlier and reached greater values in hyperimmunized animals versus normal control animals. The early edema formation was noticeable visually; however, calculations of the maximal time of response do not show highly significant differences between the mean time for hyperimmunized ($\bar{t} = 1.67 \pm 0.33$ hours) and normal ($\bar{t} = 2.29 \pm 0.18$ hours) rabbits ($P < 0.20$). The magnitude of protein leakage was significantly ($P < 0.05$) greater in the hyperimmunized group at 1 hour after injection of bacteria. Decomplemented rabbits showed significantly ($P < 0.05$) less vascular leakage than normals between 2 and 4 hours, indicating that complement does partially mediate vascular permeability in these inflammatory lesions. The role of complement probably involves the generation of anaphylatoxins C3a and C5a, which cause a direct and histamine-dependent increase in vascular leakage.[23]

The protein leakage from vessels in *E coli* lesions was almost completely abrogated in leukopenic animals. All values were significantly different from those of normal animals. The ratio of vascular permeability in lesions to permeability in control sites was close to 1; ie, no edema formed in the *E coli* injection sites of leukopenic animals. This result indicates that leukocytes are chiefly responsible for mediation of vascular permeability in *E coli*-induced lesions.

Almost absolute neutropenia was achieved in rabbits with two spaced injections of nitrogen mustard (1.5 mg/kg folowed by 1.0 mg/kg, 48 hours later). Nitrogen mustard depletes the animals of all circulating leukocytes and not exclusively neutrophils. However, on the basis of the kinetic and histologic studies

of *E coli* lesions, it appears that it is the neutropenia (rather than the leukopenia) which is responsible for inhibition of vascular permeability and partial inhibition of hemorrhage. Issekutz et al[24] have found that although the maximal rate of accumulation of monocytes in *E coli* lesions occurs between 3 and 4 hours, more than 95% of the cells entering a 3-hour-old *E coli* lesion are neutrophils (1×10^6 monocytes and 25×10^6 PMNs).[7,24] Furthermore, while almost no accumulation of neutrophils occurs in lesions more than 4–6 hours old,[7] monocytes continue to infiltrate these lesions for at least another 20 hours, becoming the histologically predominant cells after 12 hours, because PMNs start to disintegrate and monocytes continue to migrate into the lesion.[24] Vascular permeability has almost subsided and hemorrhage is almost fully developed by the time the maximal rate of influx (but not the absolute predominance) of the monocytes begins. Therefore, it seems likely that the neutrophil, which is the predominant cell type, not the monocyte, mediates the increase in vascular permeability in *E coli* lesions.

Nitrogen mustard administered systemically might be suspected of causing generalized vascular unresponsiveness towards vasoactive substances. Bradykinin and prostaglandin E_2 were injected intradermally into nitrogen-mustard-treated animals, as described in Materials and Methods, and vascular permeability and hyperemia were assessed. Bradykinin induced a threefold increase in vascular leakage over control skin sites, while PGE_2 caused a tenfold enhancement of blood flow. These values are the same as those in normal animals.[4] Thus, no unresponsiveness of the vascular bed was observed with these two vasoactive agents. Wedmore and Williams[25] further assessed the effect of nitrogen mustard on the skin of rabbits by clamping the descending aorta during the injection of the drug into the ear veins of rabbits. This procedure provided access for the drug to the skin, but not to the bone marrow of the hind legs (nitrogen mustard is known to prevent replication of stem cells in the bone marrow and thus to deplete the circulating leukocytes). The rabbits so treated responded normally to the mixture of C5a and PGE_2, thus indicating that suppression of vascular permeability due to these agents in rabbits rendered leukopenic with nitrogen mustard was not caused by any action of the drug on the skin.

The role of the neutrophil in mediating vascular permeability changes is further confirmed by the studies of Wedmore and Williams,[25] who produced edema in the skin of rabbits by injecting zymosan-activated plasma (ZAP). The active principle in ZAP is C5a des Arg, which has chemotactic but not spasmogenic activity.[26–28] ZAP in combination with PGE_2 caused vascular protein leakage, which did not occur in leukopenic animals, in keeping with the earlier described study.[15] Similar increases in vascular permeability were induced by Wedmore and Williams[25] by injections of other leukotactic substances—ie, leukotriene B_4 or the synthetic oligopeptide f-Met-Leu-Phe, in combination with PGE_2. In these studies the potentiating action of PGE_2 was needed for effective edema formation to occur. However, according to Issekutz et al,[14,15] C5a des Arg will induce vascular injury (increase in vascular permeability and hemorrhage) by itself, and in the presence of exogenous PGE there is an increase in the number of infiltrating PMNs and hemorrhage.[29]

The development of hemorrhage was not affected by hyperimmunization or decomplementation. The hemorrhage in *E coli* lesions was significantly reduced at 3 hours ($P < 0.001$) and at 6 hours ($P < 0.01$) in rabbits depleted of leukocytes. Although the amount of hemorrhage in 8-hour-old lesions was lower in leukopenic rabbits than in normal control animals, the difference was not statistically significant. The hemorrhage in 8-hour-old lesions in leukopenic rabbits could be the result of the direct action of endotoxin released from the *E coli*. Inactivation of endotoxin with mild alkali in formalin-killed *E coli* reduces the amount of hemorrhage elicited by these bacteria (unpublished observations). The hemorrhage in normal rabbits is probably mediated primarily by neutrophils, as evidenced by a decrease in hemorrhage in nitrogen-mustard-treated rabbits. Issekutz et al[14] found that injections of ZAP caused an infiltration of leukocytes during the first 4 hours after injection and extravasation of erythrocytes between 4 hours and 18 hours after the injection of ZAP. The amount of hemorrhage was closely correlated to the number of infiltrating leukocytes. There was no hemorrhage development, nor, as already mentioned, enhancement in vascular permeability in rabbits depleted of neutrophils. When the number of infiltrating PMNs was almost doubled, the later developing hemorrhage was increased about sevenfold.[29]

The involvement of neutrophils in mediation of vascular permeability and hemorrhage in *E coli* lesions seems probable in view of the data presented in this paper and the electron-microscopic observations of the lesions, in which it was found that neutrophils phagocytose the injected *E coli* and in this process degranulate.[1] The leukopenic animals, in contrast, have

been demonstrated to retain large accumulations of bacteria at the site of injection. It is of importance that in these animals the lack of phagocytosis (resulting from lack of neutrophils) is concidental with the lack of edema formation and diminished erythrocyte extravasation.

The attempts to elucidate the role of neutrophil lysosomal enzymes in the production of vascular injury include the recent report by Wasi and Movat,[30] who injected into rabbit skin substances specifically released by human neutrophils during *in vitro* phagocytosis. The vascular permeability and hemorrhage were quantitated with ^{125}I-labeled serum albumin and ^{59}Fe-labeled erythrocytes, respectively. The crude lysosomal "secretion" induced exudation of plasma and hemorrhage. Partially purified material contained several enzymes. Small doses of it injected intradermally caused only vascular permeability, whereas larger doses caused permeability and hemorrhage. Histologic examination demonstrated disruption of vessel walls and aggregation of platelets in hemorrhagic lesions.

Although the evidence pointing to the involvement of lysosomal contents is circumstantial at best, it is difficult not to correlate the changes in the PMNs, such as degranulation in the *E coli*-induced lesions, with the vascular injury that occurs once these changes have taken place. It remains to be ascertained which, if any, of the constituents of neutrophil granules mediate the vascular injury resulting in the increase in permeability and hemorrhage.

One of the conclusions drawn from the data presented concerns the sequence of events occurring in acute inflammation. The view held to date and proposed by Hurley and Spector[31] and Hurley[32,33] is that vascular permeability occurred earlier than, and was dissociated from, the infiltration of neutrophils. The data presented here show that both the kinetics of development and the extent of the response of these two parameters are closely correlated. Furthermore, complete abrogation of vascular leakage in leukopenic animals points to mediation of vascular permeability changes as well as of hemorrhage by the neutrophils.

References

1. Kopaniak MM, Issekutz AC, Movat HZ: Kinetics of acute inflammation induced by *E coli* in rabbits: Quantitation of blood flow, enhanced vascular permeability, hemorrhage, and leukocyte accumulation. Am J Pathol 1980, 98:485–498
2. Ryes MP: The aerobic Gram-negative bacillary pneumonias. Med Clin N Am 1980, 64:363–384
3. Movat HZ: The microcirculation in disseminated intravascular coagulation, Handbook of Physiology. Edited by EM Renkin, CC Michel. Washington, DC, American Physiological Society, 1982 (In press)
4. Kopaniak MM, Hay JB, Movat HZ: The effect of hyperemia on vascular permeability. Microvasc Res 1978, 15:77–82
5. Hay JB, Johnston MG, Hobbs BB, Movat HZ: The use of radioactive microspheres to quantitate hyperemia in dermal inflammatory sites. Proc Soc Exp Biol Med 1975, 150:641–644
6. Udaka K, Takeuchi Y, Movat HZ: Simple method for quantitation of enhanced vascular permeability. Proc Soc Exp Biol Med 1970, 133:1384–1387
7. Issekutz AC, Movat HZ: The in vivo quantitation and kinetics of rabbit neutrophil leukocyte accumulation in the skin in response to chemotactic agents and *Escherichia coli*. Lab Invest 1980, 42:310–317
8. Kopaniak MM, Issekutz AC, Burrowes CE, Movat HZ: The quantitation of hemorrhage in the microcirculation of inflammatory lesions and related phenomena. Proc Soc Exp Biol Med 1980, 163:126–131
9. Lillie RD: Histopathologic technique and practical histochemistry. Blakiston, New York, 1954
10. Cochrane CG, Müller-Eberhard HJ, Aikin BS: Depletion of plasma complement *in vivo* by a protein of cobra venom: Its effect on various immunologic reactions. J Immunol 1970, 105:55–69
11. Man DP, Minta JO: Immunological, structural and functional relationship between an anticomplementary protein from cobra venom *Crotalus atrox* venom and human C3. Immunology 1980, 39:503–510
12. Mayer MM: Complement and complement fixation, Experimental Immunochemistry. Second Edition. Edited by EA Kabat, MM Mayer. Springfield, Ill, Charles C Thomas, 1961, pp 133–240
13. Thomas L, Good RA: Studies on the generalized Schwartzman reaction: I. General observations concerning the phenomenon. J Exp Med 1952, 96:605–623
14. Issekutz AC, Movat KW, Movat HZ: Enhanced vascular permeability and haemorrhage-inducing activity of zymosan-activated plasma. Clin Exp Immunol 1980, 41:505–511
15. Issekutz AC, Movat KW, Movat HZ: Enhanced vascular permeability and haemorrhage-inducing activity of rabbit C5a des Arg: Probable role of polymorphonuclear leucocyte lysosomes. Clin Exp Immunol 1980, 41: 512–520
16. Johnston MG, Hay JB, Movat HZ: Kinetics of prostaglandin production in various inflammatory lesions measured in draining lymph. Am J Pathol 1979, 95: 225–238
17. Issekutz AC, Bhimji S: The effect of non-steroidal antiinflammatory drugs on *Escherichia coli* induced inflammation. J Immunopharmacol 1982, 4:11–22
18. Larsen GL, Mitchell BC, Henson PM: The pulmonary response of C5-sufficient and deficient mice to immune complexes. Am Rev Respir Dis 1981, 123:434–439
19. Tainer JA, Turner SR, Lynn WS: New aspects of chemotaxis: Specific target-cell attraction by lipid and lipoprotein fractions of *Escherichia coli* chemotactic factor. Am J Pathol 1975, 81:401–410
20. Sahu S, Lynn WS: Lipid chemotaxins isolated from culture filtrates of *Escherichia coli* and from oxidized lipids. Inflammation 1977, 2:47–54
21. Schiffmann E, Showell HV, Corcoran BA, Ward PA, Smith E, Becker EL: The isolation and partial characterization of neutrophil chemotactic factors from *E coli*. J Immunol 1975, 1831–1837
22. Wilson DM, Ormrod DJ, Miller TE: Role of complement in chemotaxis: Study of a localized infection. Infect Immun 1980, 29:8–12

23. Hugli TE, Müller-Eberhard HJ: Anaphylatoxins: C3a and C5a. Adv Immunol 1978, 26:1-53
24. Issekutz TB, Issekutz AC, Movat HZ: The *in vivo* quantitation and kinetics of monocyte migration into acute inflammatory tissue. Am J Pathol 1981, 103: 47-55
25. Wedmore CV, Williams TJ: Control of vascular permeability by polymorphonuclear leukocytes in inflammation. Nature 1981, 289:646-650
26. Fernandez HN, Henson PM, Otani A, Hugli TE: Chemotactic response to human C3a and C5a anaphylatoxins: I. Evaluation of C3a and C5a leukotaxins *in vitro* and under simulated *in vivo* conditions. J Immunol 1978, 120:109-115
27. Gerard G, Chenoweth DE, Hugli TE: Molecular aspects of the serum chemotactic factors. J Reticuloendothelial Soc 1979, 26(suppl):711-718
28. Chenoweth DE, Hugli TE: Human C5a and C5a analog as probes of the neutrophil C5a receptor. Mol Immunol 1980, 17:151-161
29. Issekutz AC, Movat HZ: The effect of vasodilator prostaglandins on polymorphonuclear leukocyte infiltration and vascular injury. Am J Pathol 1982, 107:300-309
30. Wasi S, Movat HZ: Hemorrhage and exudation in the microcirculation induced in rabbits with human neutrophil lysosomal enzymes (Abstr). Thrombos Haemostas 1981, 46:59
31. Hurley JV, Spector WG: Delayed leucocytic emigration after intradermal injections and thermal injury. J Pathol Bacteriol 1961, 82:421-429
32. Hurley JV: Substances promoting leukocyte emigration. Ann NY Acad Sci 1964, 176:918-926
33. Hurley JV: Acute inflammation: The effect of concurrent leucocytic emigration and increased permeability on particle retention by the vascular wall. Br J Exp Pathol 1964, 45:627-633

Acknowledgments

The authors wish to thank Dr. Joe O. Minta for his expert advice on the hemolytic complement assays. Mrs. Otti Freitag provided skillful technical assistance. Ms. Marica Michael provided excellent secretarial assistance.