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INHIBITION OF VASCULAR PERMEABILITY BY CYCLOHEXIMIDE IN GRANULOMATOUS INFLAMMATION

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Abstract—The effect of cycloheximide on vascular permeability in proliferative inflammation was investigated by using 8-day-old granuloma pouch induced by carrageenin in rats. The vascular permeability was assayed twice in every rat, immediately before and after the drug treatment with the aid of ^{125}I- and ^{131}I-human serum albumins (HSA) respectively. Leakage of tracers during the period of 30 min, after intravenous injection into the exudate fluid in the granuloma pouch was measured and used as an index of the vascular permeability. The ratio of the leakage values ([^{131}I]HSA/[^{125}I]HSA) was computed in order to express change of the vascular permeability induced by the drug treatment. Dose-related enhancement in the inhibition of vascular permeability by cycloheximide which was injected directly into the granuloma pouch fluid was evident over the dose range of 0.06–0.6 mg/kg. The time course of the permeability-lowering action was also investigated at a dose of 0.2 mg/kg. The maximum effect was observed at 3 hr after the injection. The permeability-lowering effect disappeared completely by 12 hr. Protein synthesis of the granuloma tissue was also inhibited and changed with time in parallel with the change in the vascular permeability. It was concluded, however, that the fall of the vascular permeability was independent from the inhibition of protein synthesis since puromycin was ineffective for lowering the vascular permeability in the dose which was strongly inhibitory for protein synthesis. The possibility of manifestation of anti-exudative effect through the stimulation of adrenal cortex was also excluded since cycloheximide showed similar activity in intact and adrenalectomized rats.

In a previous paper [1] we reported some difference between steroid and nonsteroid anti-inflammatory drugs in the mode of action on granulomatous inflammation which was provoked by carrageenin in rats. Development of granuloma tissue and exudate accumulation in the granuloma pouch were both effectively inhibited by not only steroid but also nonsteroid drugs when the treatment was started simultaneously with the provocation of the inflammation. When the drug treatment was initiated after establishment of the granuloma pouch, however, nonsteroids were ineffective for inhibiting further increase in the volume of the exudate in preformed granuloma pouch though steroid was still highly effective. Accordingly we initiated an investigation in the attempt to find nonsteroid anti-inflammatory substances in new category which would act in a similar manner as glucocorticoids for inhibiting vascular permeability in preformed granuloma pouch.

In the course of experiments along this line we examined some protein synthesis inhibitors and found that cycloheximide was markedly effective for suppressing vascular permeability of preformed granuloma pouch, while puromycin was ineffective in the dose enough for causing marked inhibition of protein synthesis in the granuloma tissue.

MATERIALS AND METHODS

Granuloma pouch. Carrageenin granuloma pouch was induced by the method of Fukuhara and Tsurufuji [1] with a slight modification. Male rats of Donryu strain, 6 weeks old and weighing 120–140 g, were used. The animals were maintained on laboratory food (Funabashi Farm, Chiba, Japan) and water *ad lib.* The rats were injected under light ether anesthesia with 8.0 ml of air s.c. on the back to make an air pouch in the shape of ellipsoid or oval. After 24 hr 4.0 ml of 2% (w/v) solution of carrageenin (Seakem No. 202, Marine Colloid Inc., Springfield, NJ, U.S.A.) in 0.9% NaCl were infused through a syringe into the air pouch already formed. The carrageenin solution was sterilized by autoclaving at 110° for 15 min and injected, after cooling, in the temperature at 40–45°. Immediately before the injection, each 0.1 mg/ml of penicillin and streptomycin was added to the solution. The day of carrageenin injection was designated as day 0. Rats bearing 8-day-old granuloma were used throughout the present experiments.

Purification of radioiodinated human serum albumin (HSA) preparations. HSA preparations labeled respectively with ^{125}I and ^{131}I were used as tracers for the assay of vascular permeability in the granuloma pouch. Through preliminary experiments commercial ^{125}I- and ^{131}I-HSA preparations were shown to contain small but significant quantity of low molecular radiochemical impurity which might interfere with the permeability measurement. Therefore, purification of the labeled HSA preparations by Sephadex G-100 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) gel filtration was performed in advance. One ml of [^{131}I]HSA (50–100 μCi, 5 μCi/mg albumin, Dainabot Co., Tokyo, Japan) or [^{125}I]HSA (50–100 μCi, 2.5 μCi/mg albumin, Kaken Kagaku Co., Tokyo, Japan) in 0.9% NaCl at pH 7.4 was loaded on a Sephadex G-100 column (12 × 300 mm) and eluted

at room temperature with 0.9% NaCl at pH 7.4 under the flow rate of 15–20 ml/hr. A fraction of the eluate corresponding to human serum albumin in the elution profile was collected and used.

Drug treatment of animals. Cycloheximide (Wako Pure Chemicals Ltd., Tokyo, Japan) and puromycin·2HCl (Makor Chemicals Ltd., Jerusalem, Israel) were dissolved in 0.9% NaCl and injected locally into the granuloma pouch fluid in the doses as shown in the section of results. In the case of puromycin, injection was performed after adjusting the pH of the solution into 7.0 with 0.2 N NaOH. The volume of the drug solution injected was adjusted to 2.0 ml/kg body wt unless otherwise stated.

Vascular permeability assay in the granuloma pouch. One μCi aliquot of the purified [^{125}I]HSA in 0.2 ml of 0.9% NaCl was injected into the femoral vein of the animals bearing granuloma pouch. After 30 min, 1.0 ml of the inflammatory exudate was withdrawn from each granuloma pouch through a syringe attached with a 1/3 mm needle and served as a sample to measure the leakage of the tracer into the pouch exudate. Immediately after the sampling of the pouch exudate, the drug to be tested was injected directly into the pouch fluid. Control animals were given the vehicle only. At an appropriate time interval after the administration of the drug, 1 μCi aliquot of the purified [^{131}I]HSA was given to the animals in the same manner as in the case of [^{125}I]HSA. Further 30 min later 1.0 ml of the pouch exudate was again withdrawn and served as a sample to measure the leakage of [^{131}I]HSA. The animals were sacrificed just after the second sampling of the pouch exudate and entire exudate in the pouch was collected to measure its vol.

Radioactivity of ^{125}I and ^{131}I was measured in an automatic well type scintillation counter Aloka JDC-751 (Nihon Musen Co., Mitaka, Japan). In the case of ^{125}I the operation mode of the counter was adjusted to the mode for selective counting of photoelectric peak of 35 keV gamma ray of ^{125}I. Radioactivity of ^{131}I was also counted similarly by selecting photoelectric peak of 360 keV gamma ray of ^{131}I. Total radioactivity of ^{125}I and ^{131}I in the entire pouch exudate of each rat was calculated and expressed in terms of the percentage of the radioactivity injected into the rat and used as an index of the vascular permeability in the granuloma pouch.

Incorporation of labeled proline into tissue non-collagen protein of the granuloma. The rats were injected 30 min before sacrifice s.c. with 15 μCi aliquot of L-[G-^{3}H]proline (63 Ci/m-mole, Daiichi Pure Chemicals Co., Tokyo, Japan) per 100 g body wt. Immediately after sacrifice the entire granuloma tissue was removed free from surrounding fat, muscle and non-granulomatous subcutaneous tissues and stored in a freezer at least for 5 weeks until use in order to make ^{131}I in the tissue decayed down to the level at which no interference with the measurement for ^{3}H took place. After thawing the granuloma tissue was minced and homogenized in a Vir-Tis 45 homogenizer at 20,000 r.p.m. for 3 min with an equal vol. of distilled water. All the procedures after thawing were carried out in 0–4° unless otherwise stated. The homogenate (4.0 ml aliquot) was added with an equal vol. of 10% trichloroacetic acid (TCA) and then the mixture was centrifuged at 3,000 r.p.m. for 5 min. The pellet, after washing twice with each 4.0 ml of 5% TCA, was resuspended in 4.0 ml of TCA and heated in a water bath at 90 for 15 min to remove nucleic acids and collagen. Thereafter, all the procedures were done at room temperature. The TCA-insoluble pellet, after washing with 5.0 ml of 5% TCA, was extracted twice with each 5 ml of ether-ethanol (1:1, v/v) mixture to remove lipid. The residue, non-collagen protein fraction of the granuloma tissue, was solubilized in 4.0 ml of 0.2 N NaOH and used as a sample for radioactivity assay and for determination of protein content. Protein determination of the sample solution was performed by Lowry's method [2] after suitable dilution.

Radioactivity assay, taking 1.0 ml of the sample solution, was carried out in a Packard Tri-Carb Model 3380 liquid scintillation counter by adding 10.0 ml of scintillation phosphor (PPO 7.0 g, POPOP 0.1 g, toluene 667 ml and Triton X-100 333 ml). Quench correction was made by channel ratio method according to external standardization procedure. Before doing quench correction, however, correction for the radioactivity of coexisting ^{125}I should be made in order to give net counts per minute (CPM) value of ^{3}H in the sample. So another 1.0 ml of the sample solution was taken for the measurement of ^{125}I in the well type scintillation counter. Conversion factor for the calculation from CPM values of ^{125}I in the well type scintillation assay into those of liquid scintillation menthod was given by the following procedure. A series of standards, consisting of each 0.05 μCi of ^{125}I, 10.0 ml of the scintillation phosphor and progressively increasing quantities of CCl_4 as a quenching substance, were prepared and counted in the liquid scintillation counter on the operation mode of ^{3}H. Channel ratio values for these standards were also obtained by external standardization procedure. On the other hand, the same quantity of ^{125}I was counted in the well type scintillation counter. The ratio of the CPM value in the liquid scintillation method to the CPM value in the well type scintillation method was plotted against the channel ratio. The calibration curve thus made was used for the correction of the radioactivity of ^{125}I in the liquid scintillation samples. The conversion factor mentioned above was obtained by feeding the calibration curve with the channel ratio value of a liquid scintillation sample.

RESULTS

Granuloma pouch holding 5–10 ml of exudate had been developed up to day 5. The vol. of the exudate increased with the passage of time reaching maximum of 20–30 ml around day 10 and then leveled off. After day 15 gradual involution of the granulomatous inflammation occurred and the exudate volume declined slowly day by day. In the present experiments, 8-day-old granuloma pouches were used, since exudative reaction at this stage had been shown to be highly active [3].

Double tracer method for evaluating drug effect. Vascular permeability in the granuloma pouch was measured twice in each rat, before and after the drug administration, with the aid of [^{125}I]HSA and [^{131}I]HSA respectively. The absolute value of the

Table 1. Double tracer method with [^{125}I]human serum albumin (HSA) and [^{131}I]HSA for evaluating drug effect on vascular permeability in granuloma pouch*

Treatment	Rat No.	Exudate vol. (ml)	Vascular permeability† (Leak of tracer) Pre-treatment ([^{125}I]HSA)	Post-treatment ([^{131}I]HSA)	Permeability change (^{131}I/^{125}I)	Relative permeability (% of control)
Control (vehicle)	1	28.18	0.70	0.69	0.99	
	3	37.66	0.81	1.05	1.29	
	5	17.05	0.26	0.34	1.30	
	7	34.05	0.73	0.64	0.88	
	9	14.90	0.26	0.27	1.04	
	11	31.44	0.42	0.32	0.76	
	13	35.75	1.37	0.97	0.71	
	Mean ± S.E.	28.43 ± 3.42	0.66 ± 0.088	0.61 ± 0.119	0.99 ± 0.089	100
Cycloheximide (0.2 mg/kg)	2	18.84	0.33	0.24	0.73	
	4	32.25	0.58	0.27	0.47	
	6	46.09	0.61	0.37	0.61	
	8	23.79	0.40	0.25	0.63	
	10	23.06	0.55	0.27	0.49	
	12	30.70	0.41	0.17	0.43	
	14	40.33	0.71	0.43	0.61	
	Mean ± S.E.	30.72 ± 3.71	0.51 ± 0.055	0.29 ± 0.032	0.55 ± 0.039	55.6

* Rats were treated with local injection into the granuloma pouch of cycloheximide (0.2 mg/kg/2.0 ml in 0.9% NaCl) or the vehicle. Assay for the leak of [^{125}I]HSA into pouch exudate was performed immediately before the injection of cycloheximide and assay for [^{131}I]HSA was done 3 hr later. Vascular permeability is expressed in terms of radioactivity which leaked into pouch exudate during the period of 30 min before the designated time.

† Figures represent radioactivity as percent of the dose of labeled HSA injected i.v.

leakage of the tracers showed significant variation among individual rats as shown in Table 1. It is inefficient, therefore, to make statistical analysis by comparing the absolute value of the leakage of [^{131}I]HSA between control and treated groups. So the ratio of the leak, [^{131}I]HSA/[^{125}I]HSA, for each rat was computed in order to express change in the vascular permeability induced by the treatment. In the control animals the ratio came near unity, since treatment with saline gave no significant influence to the vascular permeability. The ratio of the cycloheximide group was significantly lower than the control value ($P < 0.01$), indicating the inhibitory effect of cycloheximide on the vascular permeability in granulomatous inflammation. In order to indicate the effectiveness of the treatment, it is convenient to express the ratio of the leak in the treated group in terms of percent of the ratio in the control as shown in the last column of Table 1.

Dose–response relationship on the inhibitory effect on vascular permeability of cycloheximide. Based on the results of some preliminary experiments, dose–response relationship of cycloheximide in the dose range of 0.06–0.6 mg/kg was investigated. In this series of experiments the time of sampling of the pouch exudate for the assay of [^{131}I]HSA was settled at 5 hr after the drug administration. As shown in Fig. 1, vascular permeability was gradually inhibited in parallel with the logarithmical increase of the dose. Statistically significant inhibition was attained at the dose levels ranging from 0.06 to 0.6 mg/kg.

Time course of inhibitory effect of cycloheximide on vascular permeability and on [^{3}H]proline incorporation into non-collagen protein of the granuloma tissue. The dosage of cycloheximide for investigating time course of the inhibitory effect on vascular permeability and on the synthesis of non-collagen protein was settled at 0.2 mg/kg on the basis of the dose–response relationship as indicated in Fig. 1. Rats were sacrificed at intervals ranging from 2 to 12 hr after the injection of cycloheximide into the pouch. As shown in Table 2, vascular permeability in the granuloma pouch declined significantly within 2 hr and maximim inhibition was attained at 3 hr. Thereafter, the inhibitory effect disappeared by 12 hr. At 2, 3 and 5 hr the inhibition was statistically significant ($P < 0.01$).

The inhibitory effect of cycloheximide on the incorporation of labeled proline into non-collagen protein

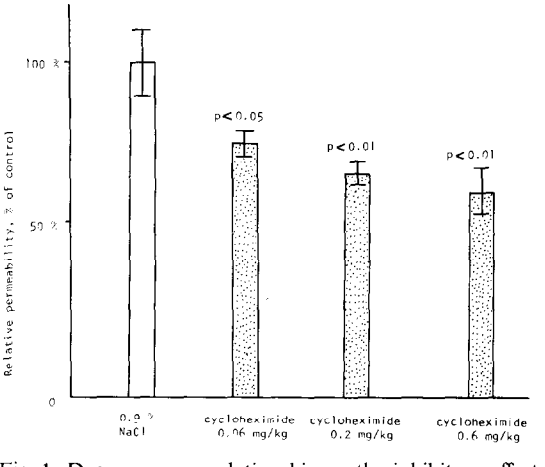

Fig. 1. Dose–response relationship on the inhibitory effect of cycloheximide (0.06–0.6 mg/kg) on the vascular permeability in granuloma pouch. Drug effect was determined 5 hr after the injection of cycloheximide. Each group consists of 7–8 rats. The vertical bar on each column represents standard error of the mean.

Table 2. Time course of inhibitory effect of cycloheximide on vascular permeability in granuloma pouch

Treatment	No. of rats	Time after treatment	Permeability change* ($^{131}I/^{125}I$)	Relative permeability (% of control)
Control	9	2 hr	1.08 ± 0.072	100
Cycloheximide (0.2 mg/kg)	6	2 hr	0.80 ± 0.092 ($P < 0.01$)	74.1
Control	7	3 hr	0.99 ± 0.089	100
Cycloheximide (0.2 mg/kg)	7	3 hr	0.55 ± 0.039 ($P < 0.01$)	55.6
Control	8	5 hr	1.08 ± 0.079	100
Cycloheximide (0.2 mg/kg)	6	5 hr	0.71 ± 0.024 ($P < 0.01$)	65.7
Control	6	12 hr	0.97 ± 0.024	100
Cycloheximide (0.2 mg/kg)	7	12 hr	0.87 ± 0.016 (not significant)	90.0

* Figures represent mean ± S.E.

fraction of the granuloma tissue changed with time in parallel with the change in vascular permeability of the granuloma pouch as demonstrated in Table 3. The maximum inhibition was shown at 3 hr, and the inhibitory effect disappeared by 12 hr. At 2, 3 and 5 hr the incorporation was significantly inhibited ($P < 0.01$).

Effect of puromycin on the vascular permeability and on the incorporation of labeled proline into non-collagen protein. In the attempt to compare the effect of cycloheximide on vascular permeability in the granuloma pouch with that of another protein synthesis inhibitor, puromycin, another series of experiments were made. Puromycin at the dose of 30 mg/kg was injected directly into the granuloma pouch and animals were sacrificed 5 hr after the injection. Control animals were injected with the vehicle only. The results are summarized in Table 4. Although puromy-

Table 3. Time course of the inhibitory effect of cycloheximide on [^{3}H]proline incorporation into non-collagen protein of granuloma tissue

Treatment	No. of rats	Time after treatment	[^{3}H]proline incorporation* DPM/μg protein	[^{3}H]proline incorporation* % of control
Control	11	2 hr	0.64 ± 0.027	100
Cycloheximide (0.2 mg/kg)	6	2 hr	0.26 ± 0.031 ($P < 0.01$)	40.6
Control	8	3 hr	0.54 ± 0.042	100
Cycloheximide (0.2 mg/kg)	6	3 hr	0.16 ± 0.013 ($P < 0.01$)	29.6
Control	8	5 hr	0.43 ± 0.056	100
Cycloheximide (0.2 mg/kg)	7	5 hr	0.16 ± 0.017 ($P < 0.01$)	37.2
Control	7	12 hr	0.44 ± 0.029	100
Cycloheximide (0.2 mg/kg)	6	12 hr	0.42 ± 0.034 (not significant)	95.5

* [^{3}H]proline (15 μCi/100 g body wt) was injected s.c. 30 min prior to sacrifice.

Table 4. Effect of puromycin on vascular permeability in granuloma pouch and on the incorporation of [^{3}H]proline into non-collagen protein of granuloma tissue

Treatment	No. of rats	Time after treatment	Permeability change* ($^{131}I/^{125}I$)	Relative permeability (% of control)	[^{3}H]proline incorporation† DPM/μg protein	[^{3}H]proline incorporation† % of control
Control (vehicle)	7	5 hr	0.95 ± 0.092	100	0.83 ± 0.060	100
Puromycin (30 mg/kg)	6	5 hr	0.83 ± 0.172 (not significant)	87.4	0.24 ± 0.017 ($P < 0.01$)	28.9
Control (vehicle)	7	5 hr	1.10 ± 0.114	100	0.51 ± 0.050	100
Puromycin (30 mg/kg)	4	5 hr	1.26 ± 0.099 (not significant)	114.5	0.17 ± 0.020 ($P < 0.01$)	33.3

* Figures represent mean ± S.E.
† [^{3}H]proline (15 μCi/100 g body wt) was injected s.c. 30 min prior to sacrifice.

Table 5. Effect of cycloheximide on vascular permeability in granuloma pouch in adrenalectomized rats*

Treatment	No. of rats	Time after treatment	Permeability change ($^{131}I/^{125}I$)	Relative permeability (% of control)
Control	8	5 hr	1.08 ± 0.11	100
Cycloheximide (0.2 mg/kg)	7	5 hr	0.74 ± 0.09 ($P < 0.01$)	68.5
Control	11	5 hr	1.13 ± 0.23	100
Cycloheximide (0.2 mg/kg)	11	5 hr	0.76 ± 0.21 ($P < 0.05$)	67.0

* Rats were adrenalectomized 24 hr prior to the measurement of the vascular permeability.

cin exerted a strong inhibitory action on the incorporation of labeled proline into non-collagen protein in the granuloma tissue, the vascular permeability in the granuloma pouch was unaffected by puromycin at all.

Effect of cycloheximide on the vascular permeability in the granuloma pouch in adrenalectomized rats. The data which appear in some reports [4–6] seem to demonstrate a possibility that cycloheximide stimulates adrenal cortex to enhance the secretion of the glucocorticoids. Therefore, we have attempted to examine whether apparent anti-inflammatory effect of cycloheximide would have been caused through stimulation of the adrenal function or not. Rats bearing 7-day-old granuloma pouch were adrenalectomized 24 hr prior to the treatment with cycloheximide. Measurement of vascular permeability was done 5 hr after the injection of cycloheximide into the granuloma pouch. The results are indicated in Table 5. Cycloheximide in the dose of 0.2 mg/kg effectively suppressed vascular permeability of the granuloma pouch in adrenalectomized rats to the same extent as in adrenal-bearing rats.

DISCUSSION

In order to evaluate potency of anti-inflammatory drugs for suppressing vascular permeability of inflamed tissues, a number of experimental models have been developed and extensively utilized in the field of experimental pharmacology [7]. In most cases of these studies drug treatment has been given prior to or simultaneously with the application of phlogistic stimuli to the local tissue. We described in a previous paper, however, that there was some difference in the sensitivity to anti-inflammatory drugs between early stage and late stage of an inflammation which was induced by carrageenin in rats [1]. When drug treatment was initiated immediately after the application of carrageenin and continued for a few days, both the exudative and proliferative reactions were inhibited markedly by both of the steroid and some nonsteroid drugs. On the other hand, when drug treatment was started after establishment of granuloma pouch tissue, the nonsteroids were ineffective, whereas steroid was still potently anti-inflammatory not only for reducing the vol. of the pre-existing exudate in the granuloma pouch but also for reducing the wet wt and the amount of several tissue components of the granuloma tissue [8, 9].

Inflammation in the type of granuloma pouch induced by carrageenin resembles synovitis in rheumatoid arthritis, as in both the cases exudate fluid accumulates in cavities encapsuled by proliferating inflammatory connective tissue. Therefore, it seems important to investigate whether or not anti-inflammatory drugs alleviate already existing proliferative inflammation. Based on this concept, we have developed a method to evaluate anti-inflammatory effect of drugs by testing the ability of suppressing vascular permeability in already established granulomatous inflammation. Double tracer technique has been introduced in the present experiment to overcome variability of the vascular permeability from animal to animal. The present method is also convenient to follow the time course of drug action, since it takes only 30 min for measuring the vascular permeability. In an attempt to follow exact time course of the effect of cycloheximide on the inflammatory tissue locus, the drug was applied directly into the granuloma pouch. Apparent parallelism in the time course of the drug effect between vascular permeability inhibition and inhibition of protein synthesis was observed.

It has been reported that in the case of some toxic substances apparent anti-inflammatory effect can be brought about as a manifestation of the toxicity [10]. LD_{50} in subcutaneous injection of cycloheximide in rats has been reported as 2.7 mg/kg [11]. In the present experiment anti-exudative effect of cycloheximide was evident in such small dose as 0.06 mg/kg and symptom suggesting the manifestation of toxicity was not detected except inhibition of protein synthesis. The profile of the time change in the inhibitory effect of the vascular permeability was essentially similar to the inhibition profile of [^{3}H]proline incorporation into non-collagen protein of the granuloma tissue. However, inhibition of protein synthesis in the granuloma tissue seems independent of the anti-inflammatory activity of cycloheximide, since puromycin, another potent protein synthesis inhibitor, was ineffective for reducing the vascular permeability in the dosage enough for inhibiting the incorporation of labeled proline into granuloma tissue protein to the same extent as cycloheximide did. The possibility of the manifestation of anti-exudative effect through the stimulation of adrenal cortex could also be excluded, since cycloheximide showed similar activity in intact and adrenalectomized rats. The results of the present experiments indicate that cycloheximide has specific anti-exudative activity in granulomatous inflammation.

Regarding the mechanism of action, interference by cycloheximide with possible chemical mediator sys-

tems in inflammation might be important. Role of histamine, serotonin and prostaglandin E as a vascular permeability-increasing factor in the granulomatous proliferative inflammation seems negative or minor as reported in previous papers [12, 13]. However, some other system such as kinins or SH-protease and vasoexin [14] may be responsible and remains for further investigation.

The vascular permeability have close correlation in general with the volume of the exudate in inflammation locus. In fact, the data of Table 1 shows a parallelism between exudate vol. and vascular permeability. Correlation coefficient with respect to the exudate volume and [^{125}I]HSA permeability calculated from the data in Table 1 is +0.62 and statistically significant ($P < 0.025$). It would be expectable, therefore, that repeated administration of cycloheximide would be therapeutically effective to yield actual reduction of the pre-existing exudate in proliferative inflammation in the cases that multiple administration of cycloheximide exerts no serious chronic or subacute toxicity.

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