To our knowledge, cardiac radiation effects in the 20,000 roentgen dose range have not been previously studied. In general, histopathologic changes seen in irradiated tissues include cellular and intercellular breakdown, as well as alterations in endothelial linings of small blood vessels(3,7). It appears that vascular damage is of fundamental importance in production of aseptic necrosis(7). In the present study, microscopic findings in the heart suggest a primary vascular lesion with secondary ischemia and infarction. The histopathologic similarity of the cardiac lesion produced by irradiation to human myocardial infarction is striking and deserves emphasis.

The application of the present technique to the experimental study of myocardial infarction is self-evident. With this technique the size and location of the myocardial necrosis could be varied by simply altering the size of the portal of radiation or appropriately positioning the animal in relation to the x-ray beam. During our study there was no difficulty in consistently irradiating the apical area of the heart. Furthermore, one may be able to vary the degree of myocardial fibrosis or necrosis by altering the total dose of irradiation given or by changing the rate at which roentgens are delivered.

Summary. The radiation effects of 2,500 to 20,000 roentgens on the canine myocardium have been studied. A collimated x-ray beam, 2.54 cm in diameter, was delivered to the heart of 12 dogs at a rate of 210 roentgens per minute. A localized area of myocardial necrosis was evident by the 7th to 10th post-irradiation day in the hearts which received a dose of 20,000 roentgens. Evolution of the myocardial lesion was documented electrocardiographically, and by pathologic examination. Microscopically, the cardiac necrosis was strikingly similar to vascular infarction in the human myocardium. The use of this radiation technique for experimental production of localized myocardial necrosis is discussed.

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Studies on Growth of Eaton's Agent in Tissue Culture.* (28206)

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Evidence that Eaton's atypical pneumonia agent is a pleuropneumonia-like organism (PPLO)(1-3) provides impetus for further investigation of biologic properties which have been difficult to determine for lack of convenient and sufficiently reproducible quantitative methods. After direct demonstration of the agent was accomplished in tissue culture(2), attention was given to possible use of this system to circumvent assay problems encountered by earlier investigators(4,5,6). This report concerns a method of quantitation based upon enumeration of Eaton PPLO colonies visualized in tissue cultures. Applications of this technique are presented in elucidating growth of the PPLO and growth inhibition by antibiotics and antisera.

Materials and methods. The Mac strain of Eaton's agent, obtained originally from Dr. Chien Liu, was passaged 8 times in chick embryos, and once in monkey kidney cell cul-

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ture. Pools of material derived contained 10^5-10^6 colony-forming units/ml, as determined by the method to be detailed. Secondary Rhesus monkey kidney cell monolayers were prepared on 6 × 22 mm flying coverslips as previously described(2), and were maintained in Eagle's minimal essential medium containing penicillin (1,000 units/ml) unless otherwise specified. Before inoculation, the culture tubes were rotated 180° to shift the coverslip away from cells attached to the tube wall; only cells on the coverslip were thereby exposed to the inocula. The indirect fluorescent antibody technique, as described elsewhere(7), was used to demonstrate the colonies and insure specificity of the results, since tissue cultures are often contaminated with PPLO. Sera employed for this purpose were obtained from a patient whose sputum yielded Eaton's PPLO (H.D.B.), and from an immunized rabbit (5). The fluorescent-stainable antibody titer against Eaton's agent(7) of the human serum was 320, and the rabbit antiserum had a titer of 5120. For the growth inhibition experiments, additional paired sera stored at −20°C from patients having atypical pneumonia in 1947-8 were made available by Dr. John H. Dingle. Antibiotics used were freshly prepared in Eagle's medium from the crystalline products supplied for bacterial sensitivity determinations by the tube method (Chas. Pfizer & Co., and Lederle Laboratories).

Results. Description and evaluation of the quantitative technique. Visualization of Eaton PPLO colonies on tissue culture monolayers(2) suggested that an assay system was feasible, resembling that used for studies of Newcastle disease virus by Wheelock and Tamm(8). In their experiments, the number of cell-infectious units of virus was estimated by counting the number of cells containing fluorescent-stainable antigen on coverslip preparations. Preliminary experiments with Eaton's PPLO revealed that the colonies were associated only with cells, and did not attach directly to the glass surfaces. While infectivity of the culture fluids could be demonstrated, it was found that growth did not occur in the medium alone. The number of organisms introduced into the system was thus reflected by the colonies present on the cell sheet.

Colonies were counted in 10 random × 400 microscopic fields of each coverslip, representing a 1% sample of the total coverslip area. From these data, the total number of colonies per unit volume of culture could be calculated. Determination of the standard deviation of this sampling method using 20 replicate tests revealed that variations ± 25% of the mean value could occur by chance (2σ). In the experiments to follow, therefore, alterations in colony counts were considered insignificant unless they exceeded the mean control values by 50%.

Growth of Eaton's agent. Studies on the growth of Eaton's PPLO in monkey kidney cell cultures were of two types. In the first experiments, evaluation was made of the time of appearance of colonies following inoculation of the cultures. A group of coverslip cultures was inoculated with the agent, and pairs were harvested after various incubation periods at 37°C. Colonies first could be seen at 2-3 days, and the maximum number per microscopic field were present at 5-8 days (Table I). Beyond this point the counts declined (Exp. A, Table I), probably due to loss of colony-bearing cells into the fluid and to development of colonies in clusters which were difficult to enumerate precisely.

In a second group of experiments, rate of development of colony-forming units was studied. A number of tissue cultures were inoculated simultaneously, and groups were harvested at intervals. Pools obtained were

TABLE I. Time of Development of Eaton PPLO Colonies in Monkey Kidney Cell Cultures.

Days post-inoculation	Avg colony count/HPF*	
	Exp A	Exp B
0	0	—
1	0	0
2	1.7	0
3	1.4	2.3
4	1.9	3.8
5	1.9	36.7
6	4.3	27.8
7	11.3	42.9
8	29.1	—
9	20.5	—
10	20.4	—

* Avg number of colonies derived from counts of 10 −×400 microscopic fields per specimen. Each experiment was performed in duplicate.

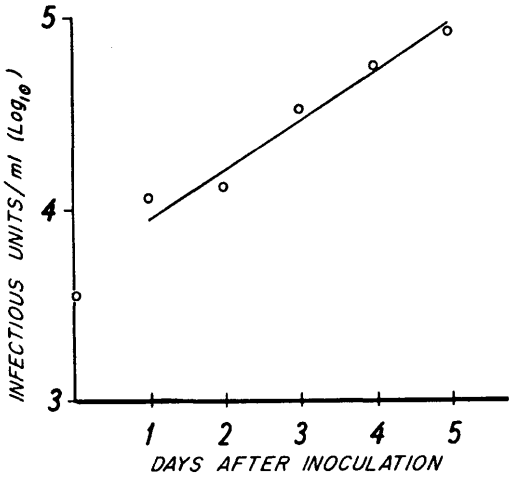

FIG. 1. Growth of Eaton's PPLO. Following inoculation of monkey kidney cell cultures, the number of organisms present at various intervals was determined by subculture and colony counts.

stored at −65°C until completion of the harvests, and were then used to inoculate duplicate coverslip cultures. After a 5-day incubation period to permit development of colonies, the coverslips were removed for counting. The data are shown in Fig. 1, following conversion of counts to the $\log_{10}$ of the number of colony-forming units/ml of culture. The points represent averages from 3 separate experiments, each performed in duplicate. Growth proceeded logarithmically during the interval from 1-5 days, and the mean generation time of the organisms was approximately 9 hours. A more rapid rise in counts has been obtained consistently during the first 24 hours, which apparently reflects a greater initial growth rate; corollary studies have indicated that this rise is not due to disaggregation of organisms.

Growth in presence of antibiotics. The technique described simplified evaluation of the effect of various antibiotics on Eaton PPLO growth. In these experiments, the fluid was removed from coverslip cultures, media containing antibiotic dilutions were substituted, and the cultures were inoculated with 10^4 colony-forming units of agent. After a 5-day incubation period, colonies were counted, and the results were expressed as complete inhibition (no growth), partial inhibition (growth up to 50% of control values), or no inhibition. The agent was resistant to penicillin, bacitracin, and polymyxin B (Table II). Streptomycin produced partial inhibition at all dilutions tested, while chloramphenicol was effective only at the higher concentrations. The agent was most sensitive to oleandomycin, tetracycline, and tetracycline derivatives, in decreasing order of relative drug effectiveness.

TABLE II. Antibiotic Sensitivity of Eaton's PPLO *in vitro*.

Antibiotic*	Final concentrations (μg/ml)† Resistant‡	Partially sensitive	Sensitive‡
Penicillin G	625	—	—
Bacitracin	23.8	238–2,380	—
Polymyxin B	10	—	—
Streptomycin	—	.1–100	—
Chloramphenicol	—	.1–1	5–10
Oleandomycin	.003	.006	.01
Tetracycline	.1	—	.5
Chlortetracycline	1.0	5–10	—
Oxytetracycline	.1	.5–1	5
Demethylchlortetracycline	.1	.5	1

* Crystalline buffered products. Concentrations tested (μg/ml): penicillin, .625-625 (1-1,000 U); bacitracin, 23.8-2,380 (1-100 U); streptomycin and chloramphenicol, 0.1-10; oleandomycin, 0.0015-10; tetracyclines, 0.05-10.

† Resistant = no effect on growth; partially sensitive = 50% or greater inhibition compared to control; sensitive = complete inhibition.

‡ Maximal drug level to which organisms are resistant, or minimal amount to which they are sensitive. Dash indicates effect not produced by levels employed.

Growth in presence of antisera. Eaton *et al* have shown that sera of patients convalescent from atypical pneumonia may neutralize the agent when cotton rats are inoculated with serum-agent mixtures(9). The variable susceptibility of cotton rats to infection has made this determination difficult, however. The method herein described provided a better defined and more reproducible system for evaluation of the growth-inhibitory effects of antisera. Two-fold serial dilutions of rabbit antiserum and human sera were prepared in tissue culture medium, and the fluid in duplicate coverslip cultures was replaced with these media prior to inoculation with 10^4 colony-forming units of agent. After 5 days, colonies were enumerated as before. Inhibition of growth was assumed when colony counts were reduced 50% or more from the values of controls which contained normal rabbit or

TABLE III. Inhibition of Eaton Agent Growth By Sera Correlated with Fluorescent-Stainable Antibody Titers.*

	Acute		Convalescent	
Serum	Inhib.	Fluor.	Inhib.	Fluor.
H.D.B.	<10	<10	320	320
2450	<10	<10	>160	2560
3091	<10	10	20	640
4335	10	20	40	320
2453	<10	<10	40	320
2417	<10	<10	>160	320
Rabbit 57	<10	<10	40	5120

* Expressed as reciprocals of the greatest serum dilutions producing significant inhibition (see text), or specific fluorescent antibody staining.

patients' acute-phase sera. The results of these experiments are compared in Table III with fluorescent-stainable antibody titrations on the sera employed. Six of the 7 pairs of sera tested demonstrated 4-fold or greater rises in inhibitory ability between the acute-phase and convalescent-phase samples, whereas all of the serum pairs had greater than 4-fold rises of fluorescent-stainable antibody.

A number of failures occurred in the first attempts to inhibit growth of Eaton's PPLO in tissue culture with antisera. It became apparent that the effect was seen only with use of large amounts of antisera (the serial dilutions above correspond to final serum concentrations in the medium of 10%, 5%, 2.5%, etc.), and that careful regulation of the inoculating dose of organisms was mandatory. Attempts to define the exact limits of this reaction using varying amounts of serum against varying amounts of agent have produced inconclusive results.

Discussion. Growth of Eaton's agent has been studied previously with the assumption that the agent was a virus(4,5,6). Now that the nature of the agent is known, assay by means of colony counting should provide greater accuracy of quantitation than the previously employed dilution-titration methods. The data presented indicate a relatively slow rate of growth compared to that of many PPLO strains, which may indicate that the culture system is deficient in, or is rapidly depleted of, needed factors. The difficulties encountered in growing Eaton's PPLO in artificial media, recently overcome by Chanock, Hayflick, and Barile(3), further emphasize the fastidious nature of the organisms.

The antibiotic sensitivity experiments support and extend studies in the literature, which were done largely by treatment of infected cotton rats or chick embryos(10,11, 12). Eaton's PPLO differs from many other types in being sensitive to oleandomycin, and in showing variable sensitivity to the tetracyclines; interpretation of these data must be tempered with a realization of the differing half-lives of the antibiotics under the experimental conditions imposed, however. Therapeutic effectiveness of a tetracycline derivative in proven Eaton PPLO infections has been demonstrated recently by Kingston, *et al.*(13).

Eaton's evidence that neutralization of the agent could be accomplished with immune sera is confirmed by the results obtained using a tissue culture system. As PPLO generally are inhibited by their specific antisera(14), these data tend further to relate Eaton's agent to atypical pneumonia. While the relative specificity of this test in comparison with the fluorescent-stainable antibody procedure has not been established, the limited data presented suggest that the neutralization technique is less sensitive.

Summary. A method for performing quantitative studies of Eaton's agent using a tissue culture system is described. The agent grew slowly in monkey kidney cell cultures, unlike many PPLO, but resembled other members of the genus *Mycoplasma* in being resistant to penicillin and sensitive to the tetracyclines. Demonstration of growth-inhibitory effects of both rabbit antiserum and sera of patients convalescent from atypical pneumonia, in confirmation of the work of Eaton, provides another parameter for studying immune response and relating Eaton's agent to human disease.

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Increased Sensitivity of Heidenhain Pouches to Exogenous Gastrin After Removal of Main Stomach.* (28207)

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Preliminary tests showed that, when exogenous gastrin is given parenterally to dogs with Heidenhain pouches, gastric secretion does not increase consistently in proportion to the dose of gastrin. When any gastric secretagogue is administered to a dog with a gastric pouch, both the main stomach and the pouch are stimulated. Acid secreted by the main stomach bathes the antrum and enters the duodenum at irregular intervals. It is possible that the responses of Heidenhain pouches to exogenous gastrin are inhibited by effects of acid in the antrum and duodenum. The present study, in which the gastric secretory responses of Heidenhain pouches to exogenous gastrin before and after total gastrectomy are compared, was performed to test this hypothesis and to seek a sensitive preparation for detection of gastrin activity in extracts of tissue.

Materials and methods. Vagally denervated (Heidenhain) gastric pouches were prepared in 4 adult mongrel dogs and studies of gastric secretion were begun 3 weeks postoperatively. The dogs were fasted for 16 hours preceding each test.

Preparation of gastrin. Gastrin was prepared by extraction of hog antral mucosa by use of stage I, with minor modifications, of a method described by Gregory and Tracy(1). Mucosa was dissected from fresh antra and divided into batches of approximately 750 g. Each batch was boiled 5 minutes in water (1 ml/g), homogenized in a large blendor (Waring), and frozen. At a convenient time later, the mixture was thawed, diluted with water (4 ml per gram of mucosa), boiled for 20 minutes, cooled, and strained through nylon chiffon gauze. The cake was discarded; the filtrate was acidified to pH 4.0 with acetic acid and the mixture was kept overnight in a refrigerator. The supernatant fluid was aspirated and discarded, and the remaining suspension was centrifuged. The precipitate was stirred mechanically for 2 hours with 4 volumes of 75% acetone containing trichloroacetic acid (TCA) in concentration of 4 g/100 ml, then the mixture was filtered through coarse filter paper coated with Hyflo.

The filtrate was extracted with ether to remove acetone and TCA, the pH was adjusted to 3.0, and residual ether was removed by evaporation in a stream of air. The mixture was then heated twice to 70°C, first at pH 5.5 and then at pH 8.5. A precipitate was separated by centrifugation and discarded. The supernatant fluid was cooled to 10°C and enough cold TCA solution (100 g/100 ml) was added to make the final concentration of TCA 4%. After standing in a refrigerator overnight, the mixture was centrifuged and the supernatant fluid, which contained

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