dition of properdin to properdin-deficient serum did not alter bactericidal effect on this organism. Thus, it would appear that *P. tularensis* is not properdin-sensitive.

Discussion. These results point to the fact that the properdin system is not involved in resistance of vaccinated or non-vaccinated humans to experimental infection by *P. tularensis*. Prechallenge properdin levels were not significantly different in volunteers who did or did not exhibit constitutional signs of disease after respiratory challenge. No significant change in properdin levels was observed following infection in 4 volunteers so studied. *P. tularensis* did not appear to be sensitive to the bactericidal effect of the properdin system *in vitro*. These findings supplement those described previously(3-7), that prechallenge agglutinin titers provided no clue as to whether the vaccinated subject would or would not become ill. Immunodiffusion studies(7) with serum samples from these same volunteers also failed to bring out a relationship between either number or pattern of precipitin lines and response of the individual after exposure. Thus, our studies have not been able to associate any particular serum component, specific or nonspecific, with immunity to experimental tularemia in volunteers.

Summary. Prechallenge properdin levels were not significantly different in 35 vaccinated or non-vaccinated volunteers who did or did not become ill after respiratory challenge with *P. tularensis*. No change was observed in properdin levels after infection in 4 volunteers so studied. *P. tularensis* was not sensitive to the properdin system *in vitro*. It would appear that the properdin system is not involved in immunity of humans to tularemia.

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Studies on Acquired Resistance of Monolayer Cell Cultures to Photodynamic Action. (27593)

ALBERT I. HOLTZ* (Introduced by C. P. Li)
Division of Biologics Standards, National Institutes of Health, Bethesda, Md.

The phenomenon of the photodynamic action of light on various biological systems has been the subject of investigation since 1899. Inhibition of photodynamic action has been described as being caused by removal of molecular oxygen, by reducing agents, and by agents which prevent the combination of the dye with the cell or other substrate(1). The photodynamic action of neutral red on cell cultures and cell culture-virus systems has been investigated in recent years in several laboratories(2,3,4). Prevention of photodynamic action in monkey kidney cell monolayers by addition of reducing agents such as cysteine and glutathione has been demonstrated by Heberling and Cheever(5). No biological system of any type was known to develop resistance to photodynamic action until Gochenour and Baron(3) reported that the photodynamic activity of neutral red-

* Present address: Nat. Heart Inst., NIH, Bethesda, Md.

stained cell cultures was lost after incubation at 36°C for 24 hours. Since the dye could be recovered from the resistant cells and was photodynamically active in fresh cells, it was concluded that the loss of photodynamic "sensitization was probably due to a change in cells rather than alteration of absorbed neutral red." Further investigation as to the nature of photodynamic resistance will be reported here.

Materials and methods. Monolayer cell cultures were grown in Pyrex Leighton tubes without cover slips. Primary rhesus monkey kidney cell cultures were used except where otherwise noted. Eagle's medium without serum or with 2% heat-inactivated calf serum was used to maintain the cells during the experiments. To eliminate ambient light, the Leighton tubes were painted with heat-resistant black enamel except for the lower part of the tube, where the cells were sheeted, which was covered with black electrician's tape. The bottom half of the tape was removed during exposure to light and all the tape was removed for microscopic examination of the cells. This method of exposure divided the cell sheet in each tube into an upper unexposed control area and a lower irradiated area. At all other times after addition of the dye the tubes were kept covered.

The cells were irradiated in a rack which held up to 13 tubes. A bank of 8 General Electric 15-watt daylight fluorescent bulbs, placed 13 cm above the cells, was used as a light source. The light source was controlled by a series 116 Powerstat which was in turn monitored by a Gossen Tri-Lux Foot Candle Meter (Model C, P. Gossen & Co., West Germany) which was placed at the level of the tissue culture cells. The light meter was calibrated with an incandescent light source of known intensity.† The tissue cultures were exposed for a period of 15 minutes at an intensity of .20 watt/cm^2. Preliminary experiments demonstrated that this exposure was followed by death of all dye-treated cells but did not kill unstained cells. The temperature of the culture tubes at the end of exposure period never rose more than 3°C above room temperature as measured by a thermometer placed in a Leighton tube in the rack.

Stock solutions of dye were prepared by dissolving the powdered dyes in sufficient boiling sterile distilled water to make a concentrate of 1:500; then were stored frozen in the dark until ready for use. Appropriately diluted dye‡ solutions were prepared immediately before each experiment, using Eagle's media and 1:500 stock solutions.

Destruction of tissue culture cells caused by irradiation was estimated by microscopic examination 24-48 hours after treatment. The amount of destruction was determined on cells in both the exposed and unexposed portions of each culture tube. The amount of cell damage was recorded as minimal (grade 1) through successive stages to death of all cells and disappearance of the cell sheet (grade 4).

Results. Induction of resistance by dyes other than neutral red. A search was made for compounds other than neutral red which might have photodynamic activity as evidenced by cellular destruction in the present monkey kidney cell culture system. Many dyes are known to have photodynamic activity in other biological systems(6).

The compound tested was considered positive for photodynamic activity if cell destruction equivalent to grade 1 or higher was noted. Unirradiated culture tubes containing dye served as controls. Direct toxicity attributable to the dye itself was determined by comparing nonirradiated cells in tubes with and without dye. Resistance to photodynamic activity was tested in separate experiments using dyes which had already been shown to be photodynamically active. In such experiments, 3 tubes were exposed to light 4 hours after addition of the dye, then

† The reference light source was kindly donated by Dr. C. W. Hiatt, Division of Biologics Standards, National Institutes of Health, Bethesda, Md.

‡ Dyes were purchased in powder form from the National Aniline Division of Allied Chemical and Dye Corp., New York, and from the Chroma-Gesellschaft Schmidt and Co., Germany, with the exception of chlorpromazine hydrochloride and prochlorperazine ethanedisulfate which were kindly supplied as the purified salt by Smith, Kline & French Co., Phila., Pa.

TABLE I. Compounds Tested for Photodynamic Activity in Primary Rhesus Monkey Kidney Cell Cultures.

Compound tested	Conc.* in Eagle's medium	Photodynamic effect (destruction of cells)	Resistance to photodynamic effect	Toxicity
Acridine orange	1:400,000	+	+	0
Azur II	1: 50,000	SL	N.T.	SL
	1:100,000	0		SL
Brilliant cresyl blue	1: 50,000	0		SL
" green†	1: 50,000	0		+
" yellow	1: 40,000	0		0
Chlorophyll (water soluble)	1: 50,000	0		0
Chlorpromazine hydrochloride	1:100,000	0		0
Eosin-y	1:200,000	0		0
Janus green B	1:150,000	0		0
Methylene blue	1: 50,000	0		0
Neutral red	1: 50,000	+	+	0
Prochlorperazine ethanedisulfate	1:100,000	0		0
Proflavine	1:400,000	+	+	0
Rhodamine B	1: 40,000	0		0
Thionin	1: 50,000	0		SL
	1:100,000	0		0
Toluidine blue	1: 75,000	+	+	0

+ = activity. 0 = no activity. SL = slight activity. N.T. = not tested.

* Highest concentration tested which did not show toxicity to the cells is reported except where toxicity is noted as present.

† Dye reduced to leuco compound when added to cells.

sets of replicate cultures to which dye also had been added at zero time were exposed at daily intervals during the following week to check on the development of resistance.

Table I summarizes the data on 16 chemicals which were tested for photodynamic activity in primary rhesus monkey kidney cell cultures. Neutral red, acridine orange, proflavine, and toluidine blue elicited photodynamic killing. Furthermore, these same dyes when left in contact with the cells from 24-48 hours produced cultures which were highly resistant to photodynamic damage. Thus, all dyes which produced definite photodynamic killing also produced resistance. Azur II showed a slight photodynamic effect but only in the toxic concentration of 1:50,000.

Attempt to isolate a resistance producing factor from resistant cells. It seemed probable that cells, when incubated with a photodynamically active dye, formed a substance which inhibited photodynamic action and thus became photodynamically resistant. If this were true it was hoped that sensitive cells could be made resistant by addition of an extract of resistant cells. Repeated attempts to isolate such a substance from resistant cells were unsuccessful. A representative experiment is described below. Four culture tubes of primary rhesus kidney cells were made resistant by incubation with 1:50,000 neutral red in Eagle's medium for 48 hours. The cells were then frozen and thawed 3 times; the resulting suspension of broken cells and dye was added to 4 new tubes containing sensitive cells. Control extracts were made from suspensions of cells which had not been incubated with neutral red, but to which a comparable amount of neutral red was added immediately before the material was mixed with susceptible cells. There were no differences in the degree of cell destruction in the cultures incubated with the 2 extracts and irradiated after 4 hours of incubation. Thus, under the conditions of this experiment, no resistance-producing factor could be transmitted.

Effect of light on development of photodynamic resistance. To determine whether light was necessary for development of photodynamic resistance, monkey kidney cultures were incubated in covered and uncovered tubes after application of 1:50,000 neutral

TABLE II. Effect of Temperature on Development of Photodynamic Resistance of Primary Monkey Kidney Cell Cultures.

Incubation prior to irradiation (hr)	Temp. of incubation 4°C	27.5°C	37°C
4	3*	3	3
24	3	1	1
48	3	1	0
72	2	1	0
96	3	1	0
120	3	1	N.S.D.
144	3	1	N.S.D.

* Denotes cell destruction following irradiation (.20 watt/cm² for 15 min.) and post-irradiation incubation for 24 hr at 37°C.

N.S.D. = Nonspecific degeneration.

red. It was observed that cells incubated in covered tubes became resistant at the same rate as those exposed to ambient light in uncovered tubes.

Effect of temperature of incubation on development of photodynamic resistance. Neutral red at a dilution of 1:100,000 in Eagle's medium was added to primary rhesus kidney culture tubes. The tubes were then divided into 3 groups and incubated at 4°C, 27.5°C, and 37°C. Three tubes were removed from each group at 4 hours and then at 24-hour intervals thereafter and exposed to light. After exposure, the tubes from each group were then placed in a 37°C incubator to determine whether any photodynamic damage would become evident.

The findings illustrated in Table II show that photodynamic resistance did not develop fully at 27.5°C and no resistance developed at 4°C in cells incubated for a total of 144 hours.

Effect of aeration of media on resistance. It has been known for some time that photodynamic action does not take place in the absence of oxygen. A similar effect was noted in cell culture tubes gassed with a mixture of 95% N_2 and 5% CO_2, 2 hours before irradiation.

The following experiment was designed to investigate whether a lowered oxygen tension in the resistant cells was the cause of the absence of photodynamic activity. Primary monkey kidney cells were made resistant by incubation with 1:100,000 neutral red for 3 days. Fresh Eagle's medium, which had been bubbled for 15 minutes with a gas mixture of 5% CO_2 in air, was added to 3 of the tubes. Three tubes received the same media unaerated, and 3 other tubes had no media change. The tubes were then incubated for 2 hours and exposed to the light source. All tubes were found to be totally resistant indicating that resistance to photodynamic action was not reversed by aeration. Therefore, resistance was probably not due to a lowered oxygen tension.

Photodynamic properties of neutral red in various cell lines. All of the cell lines tested were damaged by photodynamic action except the primary dog kidney cell cultures. Here, as elsewhere in the current work, replicate cultures of the different types of cells were held for a week and then tested for their response to the standard amount of light. The information presented in Table III indicates that none of the 3 continuous cell lines develops resistance to photodynamic damage when maintained in the presence of neutral red. In contrast, all of the primary cell cultures except dog kidney displayed resistance under these conditions.

Discussion. The mechanism by which cells develop resistance to damage by light in the presence of selected dyes is of interest. The present work fails to provide an explanation but one might hypothesize the following mechanisms. The dye might be reversibly altered by cells to an inactive compound which is attached to the photodynamically

TABLE III. Photodynamic Activity of Neutral Red 1:75,000 in Various Cell Cultures.

Cell tested	Photodynamic effect	Resistance to photodynamic effect
Continuous cultures:		
HeLa	+	—
Monkey heart	+	—
Hep-II	+	—
Primary cultures:		
Mouse embryo	+	+
Vervet monkey kidney	+	+
Rhesus " "	+	+
Rabbit kidney	+	+
Dog " *	—	—

* Dog kidney was completely resistant, *i.e.*, demonstrated no signs of photodynamic killing when irradiated at intervals from 5 hr up to 5 days of incubation.

susceptible cell sites. An alternate hypothesis is that the cells undergo a change under the influence of the dye which makes them resistant.

The discrepancy between my findings and those of Gochenour and Baron(3), who observed photodynamic resistance in continuous cell cultures, is as yet unexplained; but may be due to the differences in the method used for testing resistance. These workers used a lower light intensity and cell cultures grown in 2-ounce bottles under agar overlay.

Summary. Photodynamic destruction of monkey kidney cells by irradiation with visible light from a fluorescent light source at .20 watt/cm^2 for 15 minutes occurs 4 hours after staining with acridine orange, neutral red, proflavine, and toluidine blue. Resistance to this photodynamic effect occurs when cells stained with the same dyes are incubated in the dark for 24-48 hours prior to exposure. Lowering the temperature of incubation to 4°C inhibited the resistance forming process. A resistance-producing factor could not be obtained from resistant cells which would convert sensitive cells. Aeration of the cells or addition of fresh media does not reverse their photodynamic resistance. Thus, the depletion of a media constituent is not the cause of photodynamic resistance. Cell lines vary as to their susceptibility to photodynamic action and their ability to develop photodynamic resistance. However, under the experimental conditions outlined in this paper, primary cell cultures display resistance, whereas, continuous cell lines do not.

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Comparative Fixation of Sr^{89} and Ca^{45} by Calcified Tissues as Related to Fluoride Induced Changes in Crystallinity. (27594)

J. MENCZEL,* A. S. POSNER, H. SCHRAER,† G. PAKIS, AND R. C. LIKINS
Nat. Inst. Dental Research, N.I.H., P.H.S., U. S. Dept. of H.E.W., Bethesda, Md.

It was previously reported(1,2) that discrimination against strontium as compared to calcium, in formation of synthetic hydroxyapatite was greater in large, more slowly grown crystals than in small, more rapidly precipitated crystals. In a subsequent study of the fixation of Sr^{89} and Ca^{45} by several different tissues of the rat(3) it was shown that this discrimination increased with an increase in crystallinity as determined by X-ray diffraction line broadening analysis. Crystallinity is a measure of degree of structural perfection and size of the crystals, where more perfect and/or larger crystals are said to be more crystalline. Recent studies of rat and human bone apatite indicate that crystallinity improves with increasing incorporation of fluoride(4,5). The purpose of the present study was to investigate the crystallinity changes resulting from the long-term ingestion of fluoride in relation to the comparative metabolism of Sr^{89} and Ca^{45} in the rat.

Experimental procedure. Male Sprague-Dawley rats 35 days old were divided into 5 groups. All received the same prepared diet (6,7) containing 0.8 ppm (parts per million) fluoride. Groups were assigned drinking water containing fluoride (as NaF) as fol-

* NIH Postdoctoral Fellow from Hebrew Univ. and Hadassah Med. School, Jerusalem, Israel.

† NIDR Visiting Scientist from Penn. State Univ., State College, Pa.