Radiation, Infection, and Macrophage Function

IV. Effect of Radiation on the Proliferative Abilities of Mononuclear Phagocytes in Tuberculous Lesions of Rabbits

SAROJ CHANDRASEKHAR, KIYOSHI SHIMA, ARTHUR M. DANNENBERG, JR.,
TAKESHI KAMBARA, JACOB I. FABRIKANT, AND WILLIAM G. ROESSLER

Department of Radiological Science, Johns Hopkins School of Hygiene, and Department of Pathology, Johns Hopkins School of Medicine, Baltimore, Maryland 21205, and the Biological Science Laboratory, Fort Detrick, Frederick, Maryland 21701

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Rabbits were irradiated with 400 to 600 rads 2 to 3 days before they were infected with BCG or with virulent tubercle bacilli. Biopsies were periodically removed from the resulting lesions and incubated for 1 hr with ^3H -thymidine in vitro under hyperbaric oxygen. Twelve to 16 days after radiation there was a reduction in the percentage of mononuclear cells (macrophages and some lymphocytes) that had incorporated ^3H -thymidine in the lesions. At this time, the lesions of the irradiated group were smaller than those of controls. These results can be explained by the reduction in the number of new mononuclear cells that entered the lesions of the irradiated group and support the tenet that local mononuclear cell division occurs mainly in cells that have recently emigrated from the blood stream. An alternate, but less likely, explanation of these results would be that radiation caused the bone marrow to release a higher percentage of "defective" mononuclear cells that were unable to divide, i.e., incorporate ^3H -thymidine, in the lesions. The reduction produced by radiation in both local cell infiltration and local cell division would decrease the number of macrophages available to control the tuberculous infection in the host.

Previous reports from our laboratory showed that rabbits irradiated with 400 to 500 rads and then vaccinated intradermally with BCG had smaller lesions and smaller tuberculin reactions than controls when examined 2 to 3 weeks afterwards (4). They were smaller because fewer cells infiltrated the sites (4, 7, 11, 12). The first part of this report confirms these observations in dermal lesions and extends them to pulmonary lesions caused by BCG and virulent bacilli.

The macrophages in tuberculous lesions of the irradiated group contained more bacilli (4), which may be due to an impairment of their microbicidal and digestive abilities. It may also be due to less cell infiltration and less local cell division, both of which would decrease the number of macrophages available to handle the infection. The second part of this report shows, by means of ^3H -thymidine and autoradiography, that radiation decreases cell division within tuberculous lesions.

MATERIALS AND METHODS

Random-bred New Zealand white rabbits of both sexes, about 2.5 kg in weight, were divided into two groups. One group received whole body radiation of 400 to 600 rads (depending on the experiment) from a 2-Mev Van de Graaff unit (4). The other group served

as controls. Two to 3 days later both groups were either infected with virulent bovine tubercle bacilli (Ravenel strain) by the airborne route (2) or with BCG (Phipps strain) by the intradermal (4; Shima et al., *in preparation*; Dannenberg et al., *in preparation*) or intravenous (6; Shima et al., *in preparation*) routes (Table 1).

The Ravenel strain was cultured on Dubos-Middlebrook Tween-albumin liquid medium and aerosolized in a modified Henderson apparatus (2). The dose of bacilli inhaled by each rabbit was calculated from its weight and from samples of the aerosol collected by a liquid impinger and cultured on blood-agar (2). BCG was maintained on Lowenstein-Jensen's egg medium (4). For injection, a 6-day culture was ground in a mortar with a small amount of sodium taurocholate and suspended in 0.1% oleic albumin complex (OAC) for injection (4). The dose injected was determined by plating Lowenstein-Jensen's medium with a sample of serial dilutions made in OAC.

Tuberculin tests were performed by injecting intradermally 0.1 ml of a 1:10 dilution of Old Tuberculin. They were read 48 hr later.

Both the dermal BCG lesions and tuberculin reactions were measured with calipers (4). Length times width times double thickness times 0.2 gave their volume in cubic millimeters (4). The double thickness of the lesion was determined by subtracting the value for pinched normal skin from that of the pinched lesion.

The pulmonary lesions in 6- μ m tissue sections were measured with a 10-mm rule inserted in the 12.5 $\times$ ocular of the microscope. They were examined at 20 $\times$ magnification (10 $\times$ in the objective lens and 2.0 $\times$ in the Optovar lens). From each rabbit the diameters of 25 pulmonary lesions were tabulated, and the median of these 25 measurements was employed to represent the lesion size for that rabbit.

Biopsies of skin lesions (1 to 2 mm in thickness) were made after surrounding the lesions with 1% procaine. Biopsies of pulmonary lesions (2 to 3 mm in thickness) were taken after death by air embolism. Both types of biopsy were incubated for 1 hr at 37 C in 100% oxygen at 3 atm of pressure (3; Shima et al., *in preparation*) in the presence of 1 μ Ci of 3 H-thymidine per ml of medium 199 with 20% fetal calf serum added (3; Shima et al., *in preparation*).

The specimens were then fixed for 24 hr in 20:2:1 fixative (20 parts of 70% ethanol, 2 parts of 10% neutral Formalin, 1 part of glacial acetic acid, mixed within 1 week of use). They were stored in 70% ethanol until they were embedded in paraffin by standard histological procedures, usually after 1 week. Sections (6 μ m) made from these specimens and mounted on glass slides were dipped in Ilford K-5 (or Kodak NTB2) nuclear emulsion (3; Shima et al., *in preparation*), dried, and stored at 4 C in light-tight boxes for 3 to 5 weeks. The autoradiographs were then developed in Kodak D19 solution, fixed in Kodak acid fixer, washed with tap water, stained for acid-fast bacilli with carbolfuchsin, decolorized with acid alcohol, and counter-stained with hematoxylin. No effort was made to separate macrophages from lymphocytes, because both cell types are intimately involved in acquired resistance to tuberculosis (1, 5), and premacro-phages were morphologically indistinguishable from lymphocytes.

At 1,250 $\times$ magnification, about 1,000 mononuclear cells (macrophages plus lymphocytes) were counted in the nonnecrotic granulation tissue near the center of the lesions, and the percentage of the cells having 3 H-thymidine-labeled nuclei was determined. Cytoplasmic labeling with 3 H-thymidine was present in occasional macrophages. Possibly 3 H-thymidine was pinocytosed by the macrophages along with the serum proteins in the culture medium. Such labeling was easily distinguished from nuclear labeling.

The biopsies of the dermal lesions were small enough to show uniform 3 H-thymidine labeling in cells throughout the tissue section. The specimens containing the pulmonary lesions, however, proved too large for such uniform labeling, in spite of the fact that pulmonary tissue is spongelike. The sites selected for counting labeled cells in pulmonary tissue were therefore limited to three alveolar spaces from the cut edge of the specimen.

RESULTS

The effect of radiation on 3 H-thymidine incorporation by mononuclear cells (MN; mostly macrophages, but some lymphocytes) in tuberculous lesions was evaluated in seven experiments: four with dermal BCG lesions, one with pul-

monary lesions caused by the inhalation of virulent bovine-type tubercle bacilli, and two with pulmonary lesions caused by the intravenous injection of BCG (Table 1).

Nine to 16 days after radiation, the dermal BCG lesions and tuberculin reactions in the irradiated rabbits were smaller than those in the unirradiated controls (Table 2). The pulmonary lesions of the two groups showed similar differences (Table 2). These findings indicate that radiation reduced MN infiltration during the preceding period, as the number of MN per unit volume of BCG lesion was the same in irradiated and control rabbits (4).

Twelve to 16 days after whole body radiation there was a decrease in the incorporation of 3 H-thymidine by MN in tuberculous lesions produced by both BCG and virulent bacilli (Table 3). The decrease in 3 H-thymidine incorporation at 2 weeks was statistically significant in five of the seven experiments, and the other two experiments showed the same trend.

Five weeks after radiation a significant increase in 3 H-thymidine incorporation by MN occurred in the dermal BCG lesions of two experiments, but the reverse occurred in the dermal lesions of a third experiment (Table 3). Some of the variations observed among these experiments may be due to differences in the dose of live and dead bacilli injected and seasonal changes in the rabbit stock.

The MN incorporating 3 H-thymidine were in the peripheral areas of the lesion. The MN in the centers were usually necrotic, and many near the centers were undoubtedly dying. These findings are consistent with the interpretation that newly arrived MN were the cells that incorporated 3 H-thymidine.

3 H-thymidine-labeled MN rarely contained tubercle bacilli. Only one or two such cells were found in all the experiments herein reported. This finding has two interpretations. (i) The presence of intracellular tubercle bacilli inhibited MN division or (ii) intracellular bacilli were present only in older, more centrally located MN which do not divide.

Biopsies of 48-hr tuberculin reactions were made in two experiments. Between 1 and 3 weeks, the number of MN present in these reactions was reduced by radiation, since these reactions were smaller in the irradiated group (Table 2; reference 4). However, the percentage of 3 H-thymidine-labeled MN was not changed (Table 3). These results are explained below.

DISCUSSION

In a previous report from our laboratory, MN (mostly macrophages) in BCG lesions of

TABLE 1. *Experimental data*

Expt	No. of rabbits ^a	Dose and type of live bacilli injected or inhaled ^b	Opacity of the bacillary suspension injected ^c	Whole body dose of radiation (rads)	Interval between radiation and bacillary injection or inhalation (days)	Total leukocyte count ^d
1	C, 6 R, 5	0.1 × 10 ⁶ BCG in each of 8 id sites	0.400	400	2	8,800 ± 600 1,800 ± 100
2	C, 6 R, 7	9 × 10 ⁶ BCG in each of 10 id sites	3.050	400	2	11,600 ± 600 3,300 ± 400
3	C, 3 R, 4	60 × 10 ⁶ BCG in each of 6 id sites	1.080	500	2	11,100 ± 1,300 3,700 ± 500
4	C, 8 R, 7	15 × 10 ⁶ BCG in each of 8 id sites	0.690	500	2	6,600 ± 500 1,800 ± 300
5	C, 5 R, 5	5.6 × 10 ⁶ virulent bovine-type bacilli (Ravenel strain), resp route		500	3	8,400 ± 1,200 2,100 ± 300
6	C, 6 R, 10	4,000 × 10 ⁶ BCG iv	1.500	600	2	7,700 ± 400 2,200 ± 200
7	C, 6 R, 10	600 × 10 ⁶ BCG iv	1.300	600	2	9,200 ± 700 1,900 ± 200

^a Control, C; irradiated, R.

^b Route of bacillary administration: id, intradermal; resp, respiratory; iv, intravenous.

^c Opacity of the bacillary suspension is the optical density reading made in a 1.25-cm test tube in a Bausch & Lomb Spectronic-20 spectrophotometer at 540 nm. It is an index of the total number of (live plus dead) bacilli injected.

^d Original and 2 to 3 days after radiation. The means and their standard deviations are listed.

irradiated rabbits, 2 weeks after radiation with 400 to 500 rads, were shown to contain more bacilli than those of unirradiated controls (4). Their increased bacillary content may be due to less MN local division and infiltration, or it may be due to deficient MN bactericidal or bacteriostatic capacity, or both. The studies reported herein employed ³H-thymidine to elucidate further the effect of whole body radiation on tuberculosis.

Biopsies of tuberculous lesions and tuberculin reactions were incubated *in vitro* with ³H-thymidine under hyperbaric oxygen. Such *in vitro* incubation eliminated the possibility that the cells studied were labeled elsewhere [e.g., in the bone marrow (10; A. Volkman, Ser. Haematol., *in press*) or in the blood stream] and then migrated into the local lesion.

Twelve to 16 days after radiation with 400 to 600 rads, a decreased percentage of ³H-thymidine-labeled MN was present in tuberculous

lesions of most experiments (Table 3). At this time, the number of infiltrating MN was reduced by radiation (Table 2; references 4, 7, 11, 12), because the production of macrophages by the bone marrow was reduced (8, 12). It is mainly recent immigrants that divide locally (8, 9; Shima et al., *in preparation*; Dannenberg et al., *in preparation*), and a reduction in the number of such immigrants would leave an older resident population with less propensity for division. Therefore, the radiation-produced reduction in the ³H-thymidine labeling index of local MN could be due to the decreased infiltration of new cells and not due to a decreased mitotic rate of the local residents.

The MN released by bone marrow 2 weeks after radiation may be normal in their capacity to divide in inflammatory sites. In 48-hr tuberculin reactions elicited at this time, the ³H-thymidine labeling index of local MN was the same in both irradiated and control rabbits.

TABLE 2. *Size of tuberculous lesions and tuberculin reactions of irradiated and control rabbits at various times after infection*

Expt ^a	Age of lesion when biopsied (days)	Tuberculous lesions ^b			Tuberculin reactions at 48 hr ^b		
		Control	Radiated ^c	P values ^d	Control	Radiated ^c	P values ^d
1, BCG id	7	6 ± 2	5 ± 2		0	0	
	12	16 ± 4	4 ± 2	0.02	23 ± 6	5 ± 5	0.03
	19	37 ± 7	18 ± 8	0.06	217 ± 27	79 ± 18	0.001
	33	13 ± 3	9 ± 7		235 ± 87	167 ± 36	
2, BCG id	7	118 ± 20	66 ± 9	0.02	24 ± 6	5 ± 3	0.008
	12	165 ± 20	94 ± 24	0.02	264 ± 60	141 ± 37	0.05
	19	118 ± 14	104 ± 18		262 ± 33	389 ± 74	0.07
	33	42 ± 7	34 ± 5		358 ± 27	364 ± 57	
3, BCG id	7	112 ± 39	42 ± 9	0.07	3.2 ± 1.2	1.4 ± 0.6	0.02
	14	239 ± 72	119 ± 20	0.09	465 ± 20	272 ± 58	0.002
	21	257 ± 94	172 ± 24		689 ± 175	349 ± 89	0.01
	35	91 ± 30	76 ± 13		356 ± 87	377 ± 155	
4, BCG id	7	39 ± 5	17 ± 3	0.001	0	0	
	12	61 ± 9	24 ± 7	0.004	174 ± 41	42 ± 20	0.01
	18	117 ± 20	81 ± 22	0.12	606 ± 92	206 ± 66	0.001
	25	128 ± 24	90 ± 35		917 ± 272	474 ± 139	0.08
	34	47 ± 7	35 ± 5	0.09	736 ± 166	450 ± 218	
5, Virulent bacilli resp route	13	2.5 ± 0.1	2.0 ± 0.1	0.004			
6, BCG iv	10	2.8 ± 0.1	2.5 ± 0.1	0.03	0	0	
7, BCG iv	12	2.9 ± 0.1	2.5 ± 0.1	0.007	42 ± 18	6 ± 2	0.03

^a Abbreviations: id, intradermal; resp, respiratory; iv, intravenous.

^b Size of the dermal lesions is listed as a volume in cubic millimeters. The size of the pulmonary lesions is listed as a diameter in millimeters × 20. The means and their standard deviations are listed.

^c Whole body radiation was given 2 or 3 days before the infections were started.

^d P values are given for results that were sufficiently different to warrant statistical treatment.

Since every MN had entered these reactions during the preceding 48 hr, every MN present was a recent immigrant. These results support the suggestion that the population of MN entering lesions 2 weeks after radiation was normal in its ability to divide and did not contain an additional percentage of MN that were "defective" in incorporating ³H-thymidine.

The data presented in Tables 2 and 3 provide additional evidence that recent MN immigrants are the cells that divide in situ. In BCG lesions 12 to 16 days after radiation, when the growth of the lesion indicated that the number of entering MN was low, the percentage of ³H-thymidine-labeled MN was low; i.e., the labeled cells made up a smaller proportion of the cell population. At this time in 48-hr tuberculin reactions of the irradiated group, the proportion of ³H-

thymidine-labeled MN was not reduced because every MN had recently entered the reaction and therefore the entire population, although reduced in size, was made up of new cells.

In two experiments 5 weeks after radiation, there was more ³H-thymidine incorporation by MN in the dermal lesions of the irradiated group. At this time, these lesions may have contained a higher proportion of recent immigrants (7), in compensation for the reverse effect 3 weeks earlier when fewer MN were present to control the infection.

To recapitulate, at the time when whole body radiation had the most effect on host resistance to tuberculosis, radiation decreased the number of MN in tuberculous lesions and also the percentage of these cells that incorporated ³H-thymidine. One interpretation of this result

TABLE 3. *Percentage of mononuclear cells in biopsies of tuberculous lesions and tuberculin reactions of irradiated and control rabbits that incorporated ^3H -thymidine during 1 hr of incubation in vitro*

Expt ^a	Age of lesion when biopsied (days)	Percentage of ^3H -thymidine labeled mononuclear cells ^b					
		Tuberculous lesions			Tuberculin reactions at 48 hr		
		Control	Radiated ^c	<i>P</i> values ^d	Control	Radiated ^c	<i>P</i> values ^d
1, BCG id	7	2.2 $\pm$ 0.5	2.4 $\pm$ 0.4				
	12	5.0 $\pm$ 0.8	2.5 $\pm$ 0.3	0.005	1.2 $\pm$ 0.3	1.2 $\pm$ 0.6	
	19	2.8 $\pm$ 0.2	2.4 $\pm$ 0.8		3.4 $\pm$ 1.5	4.4 $\pm$ 1.3	
	33	2.1 $\pm$ 0.2	3.8 $\pm$ 0.5	0.006	3.7 $\pm$ 1.3	3.1 $\pm$ 1.3	
2, BCG id	7	1.8 $\pm$ 0.3	2.0 $\pm$ 0.6		3.3 $\pm$ 0.7	2.4 $\pm$ 0.4	
	12	2.1 $\pm$ 0.6	1.8 $\pm$ 0.5		4.2 $\pm$ 0.6	4.6 $\pm$ 1.6	
	19	0.9 $\pm$ 0.2	1.6 $\pm$ 0.3	0.04	1.2 $\pm$ 0.4	2.1 $\pm$ 1.4	
	33	1.1 $\pm$ 0.2	1.4 $\pm$ 0.3		2.6 $\pm$ 0.6	1.3 $\pm$ 0.4	0.06
3, BCG id	7						
	14	3.6 $\pm$ 1.7	1.8 $\pm$ 0.5	0.17			
	21	4.2 $\pm$ 1.6	1.4 $\pm$ 1.2	0.11			
	35	3.6 $\pm$ 0.7	6.3 $\pm$ 0.9	0.03			
4, BCG id	7	0.6 $\pm$ 0.2	0.3 $\pm$ 0.1	0.11			
	12	1.3 $\pm$ 0.3	0.3 $\pm$ 0.1	0.004			
	18	1.2 $\pm$ 0.2	0.3 $\pm$ 0.1	0.001			
	25	1.0 $\pm$ 0.1	0.2 $\pm$ 0.1	0.001			
	34	1.5 $\pm$ 0.2	0.3 $\pm$ 0.1	0.001			
5, Virulent bacilli resp route	13	3.6 $\pm$ 0.9	1.3 $\pm$ 0.6	0.04			
6, BGG iv	10	2.5 $\pm$ 0.6	1.1 $\pm$ 0.4	0.03			
7, BGG iv	12	2.1 $\pm$ 0.6	0.9 $\pm$ 0.1	0.05			

^a Abbreviations: id, intradermal; resp, respiratory; iv, intravenous.

^b The means and their standard deviation are listed. Unfortunately, no biopsies of the tuberculin reactions in experiments 3 and 4 were taken for in vitro ^3H -thymidine incorporation. At the time of these experiments, we did not realize the importance of the identical results in the control and irradiated rabbits of experiments 1 and 2.

^c Whole body radiation was given 2 or 3 days before the infections were started.

^d *P* values are given for results that were sufficiently different to warrant statistical treatment.

would be that radiation caused the bone marrow to release a higher proportion of MN incapable of division in the local inflammatory site. This interpretation was not supported by our studies on labeled MN in tuberculin reactions. A more likely interpretation would be that radiation reduced MN production in the bone marrow and that its effect on local MN division was due to the resulting diminished supply of recent immigrants, which are the main MN dividing in the local lesion.

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