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Response of Sublethally Irradiated Monkeys to a Replicating Viral Antigen

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Temporal effects of exposure to sublethal, total-body X radiation (400 R) on responses to vaccination with the attenuated Venezuelan equine encephalomyelitis vaccine virus, TC-83, were examined in rhesus monkeys. Viremia, often with delayed onset, was prolonged even when irradiation preceded vaccination by 28 days. Virus titers were increased, particularly in groups irradiated 4 or 7 days before vaccination. Delay in appearance of hemagglutination-inhibition and serum-neutralizing antibody correlated closely with persistence of viremia in irradiated-vaccinated monkeys. The temporal course of antibody response was markedly affected by the interval between irradiation and injection of this replicating antigen. With longer intervals between irradiation and vaccination, the somewhat depressed antibody responses approached normal or surpassed those of nonirradiated monkeys. Vaccination 14 days after radiation exposure resulted in lethality to 8 of 12 monkeys, apparently as a result of secondary infection. The additional lymphopenic stress due to the effect of TC-83, superimposed on the severely depressed hematopoietic competence at 14 days, undoubtedly contributed to this increased susceptiblility to latent infection.

After various immunosuppressive measures, both potentiation (9, 14, 16, 19, 20, 34, 35) and nonpotentiation (11, 13, 20, 22) of experimentally induced virus infections have been described, but these phenomena have received little attention in irradiated monkeys. The most pertinent study of Syverton et al. (31) revealed decreased resistance of irradiated monkeys to infection with poliomyelitis virus, with earlier onset and longer persistence of viremia and increased fatality rates. In other related studies, the appearance of circulating antibody was delayed in irradiated monkeys immunized with either typhoid vaccine or sheep erythrocytes (23), findings consistent with responses observed in analogous studies with rodents (25, 30, 32). Another report (4) described enhancement of the primary humoral antibody response against sheep erythrocytes in irradiated rhesus monkeys.

Studies with mice given attenuated Venezuelan equine encephalomyelitis (VEE) vaccine virus, TC-83, indicated (29) that prior exposure to sublethal irradiation delayed but did not suppress development of either antibody or specific resistance, and that as the interval between irradiation and vaccination increased, the time required for development of protective immunity decreased. The data of Reynolds et al. (21) suggested a similar delay or depression in the primary response of hemagglutination-inhibition (HI) antibody in rhesus monkeys irradiated 24 h before VEE immunization. However, the latter study did not examine the temporal relationship of irradiation to vaccination (21).

Because of the increased use of live vaccines to control viral infections, the temporal relationship between irradiation and live vaccination in monkeys was of particular interest. Further, TC-83, developed as a vaccine for human use (1) and subsequently used in animals during recent epizootics (27, 28), was selected because prior studies in mice suggested that there may be a close correlation between persistence of this live, viral antigen and the primary humoral antibody response of an irradiated host (29).

MATERIALS AND METHODS

Animals. Healthy, young, mature rhesus monkeys (*Macaca mulatta*) of both sexes, weighing 2.5 to 4.0 kg, obtained from commercial sources, were used. Monkeys were housed in individual cages, fed a commercial ration supplemented with fresh fruit, and provided with water ad libitum. Absence of circulating antibodies for VEE was confirmed before monkeys were assigned to the study.

Irradiation. Total-body irradiation exposure, 400 R, was delivered with a 1 MeV, 3 mA, X-ray generating unit, target-to-midline distance 150 cm, HVL 2.74-mm lead, at an exposure dose rate of 30 R/min. Monkeys tranquilized with ketamine hydrochloride

were irradiated while in plastic cylinders rotated at 2 rpm. Exposure dose was measured in air with a recently calibrated Victoreen condenser R-chamber.

Virus. The attenuated, live VEE virus, TC-83, has been described previously (3, 18). TC-83 vaccine, lot 3 (Merrell-National Laboratories, Division of Richardson-Merrell, Inc., Swiftwater, Pa.) was reconstituted with 1.2 ml of Hanks balanced salt solution. Monkeys were vaccinated subcutaneously with a 0.4-ml dose containing approximately 5×10^4 plaque-forming units (PFU) of virus as titrated with Vero cell cultures.

Experimental design. Three replicate experiments were conducted. In each experiment, monkeys were inoculated with TC-83 vaccine on the same day; consequently, irradiation was accomplished at varying times before inoculation. Monkeys were allocated randomly into six experimental groups, a vaccine control (VC) group and a radiation control (RC) group; experimental groups were irradiated at 2 h or 1, 4, 7, 14, or 28 days before vaccination (R-2h, R-1, R-4, R-7, R-14, R-28). All monkeys were bled by femoral venipuncture for 5 consecutive days before irradiation to establish base-line values for complete blood count, hematocrit, and platelets. After irradiation, blood samples were collected periodically to monitor hematopoietic depression.

After vaccination, monkeys were bled every 8 h for 3 days, daily for the next 18 days, twice a week for 4 weeks, and once a week for 4 additional weeks to collect serum and whole blood. Bleedings were done at the same time each day. All sera were stored at −70 C until assayed.

Viremia assay. Sera were assayed for virus content with Vero cell cultures by a microtiter method (8). The dilution of serum that resulted in a countable number of plaques was used to estimate virus titer.

HI test. Serum titrations for VEE HI antibodies were performed by the method of Clarke and Casals (5) as modified for microtiter by Sever (24).

Serum neutralization test. For assay of serum-neutralizing antibody, the method of Earley et al. (8), modified for microtiter, was used with Vero cell cultures. Serum was inactivated at 56 C for 30 to 45 min and serial fivefold dilutions, starting with an initial ilution of 1:20, were prepared in Earle 199 medium. One-tenth milliliter of the appropriate serum dilution was incubated overnight at 4 C in a microtiter tray with 0.1 ml of TC-83 dilution that contained approximately 200 PFU. One-tenth milliliter of this mixture was transferred onto a monolayer culture of Vero cells. Cultures were incubated for 1 h at 37 C before addition of a 0.33% Ionagar overlay. After additional incubation at 37 C for 72 h, the agar overlay was decanted and the cell sheet was fixed with 95% ethyl alcohol and stained with crystal violet. The highest serum dilution yielding 80% plaque reduction was selected as the end point for neutralizing antibody. Plaque reduction neutralization (PRN) titer is reported as the geometric mean end point for tests performed in triplicate.

Normalized lymphocyte values. Five consecutive, daily, preirradiation, absolute lymphocyte counts were determined to establish a mean base-line lymphocyte value on each monkey. Succeeding peripheral lymphocyte counts were divided by the corresponding mean base-line count; a group mean normalized lymphocyte value was obtained from them for each group of monkeys. A normalized value of 1.0 denoted no change from base line.

Statistics. Mean viremia and antibody titers are presented as geometric mean values. All statistical comparisons are based on the unpaired Student's t test statistic applied to log-transformed data.

RESULTS

Infection with TC-83 virus. Time of appearance, duration, and peak titer of virus in samples of peripheral blood were determined for nonirradiated and irradiated groups of monkeys (Table 1). Only five of eight nonirradiated VC monkeys developed detectable levels of viremia ($>10^2$ PFU/ml); the first positive samples were recovered at 40 to 64 h postinoculation. Viremia was of brief duration, never more than 32 h, but was variable in magnitude; maximum titers ranged from 440 to 40,000 PFU/ml.

In contrast, except for three monkeys irradiated 28 days before vaccination, no. R-28-F, G, and H, all irradiated-vaccinated monkeys became viremic (Table 1). In some, however, particularly in the group irradiated 1 day preinoculation, development of detectable viremia was delayed; in one monkey (no. R-1-D), it was delayed for as long as 11 days. Persistence of viremia was prolonged in all irradiated groups; the greatest prolongation, 8 to 9 days, occurred in groups irradiated 1 or 4 days before vaccination. A bimodal pattern with two viremia peaks, often separated by several days, was observed in 13 of 37 irradiated monkeys. Maximum viremia titers in the 4- and 7-day irradiation groups were extremely variable, but their group means were significantly higher ($P < 0.05$) than the mean titer for the VC group. Unlike the three monkeys in the VC group that failed to develop detectable viremia, the three nonviremic monkeys in the 28-day group also failed to develop specific antibodies.

Prolonged viremia probably was not associated with delayed clearance of the viral inoculum by the reticuloendothelial system (RES) because vascular clearance of 1 ml (10^9 PFU) of TC-83 was not significantly different for irradiated and nonirradiated monkeys (12). Likewise, interferon was not detectable in a few selected serum samples obtained from irradiated monkeys during their viremic period (Hilmas and McManus, unpublished data).

Vaccination of nonirradiated monkeys or total-body radiation exposure of nonvaccinees caused no fatalities. Death did occur, however, in some irradiated-vaccinated groups: two of four in the R-2h group, one of nine in the R-1

TABLE 1. *TC-83 viremia pattern and peak viremia titers (PFU/ml) for individual monkeys in each group*

Group[a]	Monkey	TC-83 viremia by day postvaccination	(×) Peak titer (PFU/ml 10^3)
		1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16	
VC	A	Not detected	0.05[b]
	B	Not detected	0.05[b]
	C	Not detected	0.05[b]
	D	×	0.
	E	×	23.
	F	×	0.
	G	×	40.
	H	×	3.
		Group mean	0.73[c]
R-2h	A	×	11.
	B	×	47.
	C	×	3.6
	D	×	20.
		Group mean	14.[c]
R-1	A	×	0.29
	B	×	0.22
	C	×	1.7
	D	×	1.0
	E	×	2.0
	F	×	2.1
	G	×	1.5
	H	×	2.0
		Group mean	1.0[c]
R-4	A	×	39000.
	B	×	280.
	C[d]	×	80.
	D	×	0.14
	E	×	2.4
	F	×	400.
	G	×	2.4
	H	×	23.
	I	×	51.
		Group mean	41.[c,e]
R-7	A	×	200.
	B	×	0.67
	C	×	80.
	D	×	60.
	E	×	1.0
	F	×	640.
		Group mean	27.[c,e]
R-14	A	×	0.50
	B	×	3.5
	C	×	4.0
	D	×	43.
	E	×	95.
		Group mean	7.8[c]
R-28[f]	A	×	0.50
	B	×	1.1
	C	×	0.30
	D	×	2.0
	E	×	0.13
		Group mean	0.53[c]

[a] VC, Vaccine controls, (R-2H), (R-1), (R-4), (R-7), (R-14) and (R-28), irradiated 2 h, or 1, 4, 7, 14, or 28 days before vaccination, respectively.

[b] Assigned values which are one-half of the minimum detectable serum virus levels.

[c] Geometric mean titers for each group.

[d] R-4-C had two viremic peaks with the same titer.

[e] $P < 0.05$ when compared to VC group.

[f] R-28-F, G and H not included in Table 1 since they were not detectably viremic and had no detectable HI or serum-neutralizing antibody in their sera.

group, and eight of twelve in the R-14 group. Regardless of time of death, clinical signs attributable to VEE infection were absent, but evidence of *Streptococcus pneumoniae* infection was observed in several monkeys on postmortem examination.

Pheripheral lymphocytes. Both vaccination alone (VC) and irradiation alone (RC) induced lymphopenic responses (Fig. 1A). A marked lymphopenia to 10 to 20% of normal levels developed within 24 to 48 h and was maintained for 17 to 20 days after 400 R of total-body irradiation exposure. Likewise, in nonirradiated monkeys vaccinated with TC-83, a slight decrease in total peripheral lymphocytes consistently occurred within 3 to 5 days, but was of lesser degree, generally 75 to 80% of normal levels.

The extent and duration of lymphocyte depression in all RC and irradiated-vaccinated monkeys were similar during the first 20 days after irradiation (Fig. 1A, B, C). Subsequently, the recovery pattern for lymphocyte response depended upon the temporal relationship of irradiation to vaccination. Monkeys irradiated 4 days before vaccination exhibited a lymphocyte response pattern similar to that of the RC group (Fig. 1a), whereas monkeys in the R-1 and R-7 groups showed earlier recovery of peripheral lymphocytes (Fig. 1B, C). In contrast, lymphocyte recovery patterns for monkeys in the R-14 and R-28 groups remained somewhat depressed for as long as 50 to 60 days postirradiation (Fig. 1B, C). Monkeys in the R-14 group that died within 20 days postvaccination generally showed no evidence of significant recovery of lymphocyte counts and many manifested a gradual decrease to below 0.1 to 0.2 of normal.

Antibody responses. Except for three previously described monkeys in the R-28 group, all surviving vaccinees developed HI and neutralizing antibodies. Generally, antibody was detected within 1 to 3 days after termination of detectable viremia, and neutralizing activity appeared 1 to 2 days before HI antibodies, possibly reflecting the 10-fold greater sensitivity of the PRN assay.

Comparisons of group mean values for HI response patterns are shown in Fig. 2 and for PRN response patterns in Fig. 3. Detectable antibody appeared more slowly in irradiated-vaccinated groups than in the VC group; delays of 3 to 5 days in the initial appearance of neutralizing antibody and of 4 to 7 days in that of HI antibody were observed. Temporal differences in time of appearance for group responses appeared to be related to mean time of maximum viremia as well as to mean duration of viremia. The shortest delay for both antibody activities (3 and 4 days, respectively) occurred in the R-7 group. Except for the HI response of group R-28, the rate of increase for both activities was somewhat slower for irradiated-vaccinated groups than for the VC group, resulting in a delay in achieving maximum antibody titers. The maximum mean HI titer occurred on day 15 after vaccination for the VC group, and on day 18 for group R-28, but was 2 to 3 weeks later for groups R-4, R-7, and R-14, and 5 to 6 weeks later for group R-1.

Unlike the relatively short-lived peak responses for VC, R-4, and R-7 groups, titers for R-1, R-14, and R-28 remained at maximum levels for at least 6 weeks. The time for maximum neutralizing activity was delayed for all irradiated-vaccinated groups; maximum titer was achieved on day 17 for VC monkeys, 2 to 3 weeks later for groups R-1, R-4, and R-7, and 5 to 7 weeks later for R-14 and R-28. There was suggestive evidence for a biphasic PRN response for VC monkeys with a second maximum approximately 4 weeks after the first. Response patterns for irradiated-vaccinated groups showed no biphasic trend; R-1, R-4, and R-7 patterns gradually decreased from peak values, and R-14 and R-28 responses remained at approximately maximum levels until termination of the study.

DISCUSSION

Our findings with monkeys exposed to 400 R of sublethal, total-body X-irradiation from 2 h to 28 days prior to vaccination with the attenuated TC-83 strain of VEE clearly indicate that the temporal relationship between irradiation and vaccination has a significant effect on development, magnitude, and persistence of viremia and on antibody response patterns. Although parameters of the viremic response are accentuated in all irradiated groups, delays in onset of detectable viremia, persistence of virus and time required for development of maximum virus titers in the blood are particularly notable in groups irradiated 1 or 4 days before vaccination, and maximum virus titers, in the 4- and 7-day preirradiated groups.

In contrast, detectable viremia failed to develop in three of eight monkeys in both the VC and R-28 groups. Unlike nonviremic monkeys in the control group, however, the corresponding irradiated monkeys failed to develop an antibody response. It is highly unlikely that error in administration of vaccine was responsible because nonreactors were observed in each R-28 replicate study. Similar defects in antibody synthesis have been attributed to insufficient virus replication or to induction of immune tolerance (7). Neither explanation is fully acceptable for these monkeys. Failure of virus to replicate would imply that during recovery from irradiation damage, mechanisms responsible for viral clearance and degradation are in a hyperactive state; viremic and antibody responses of

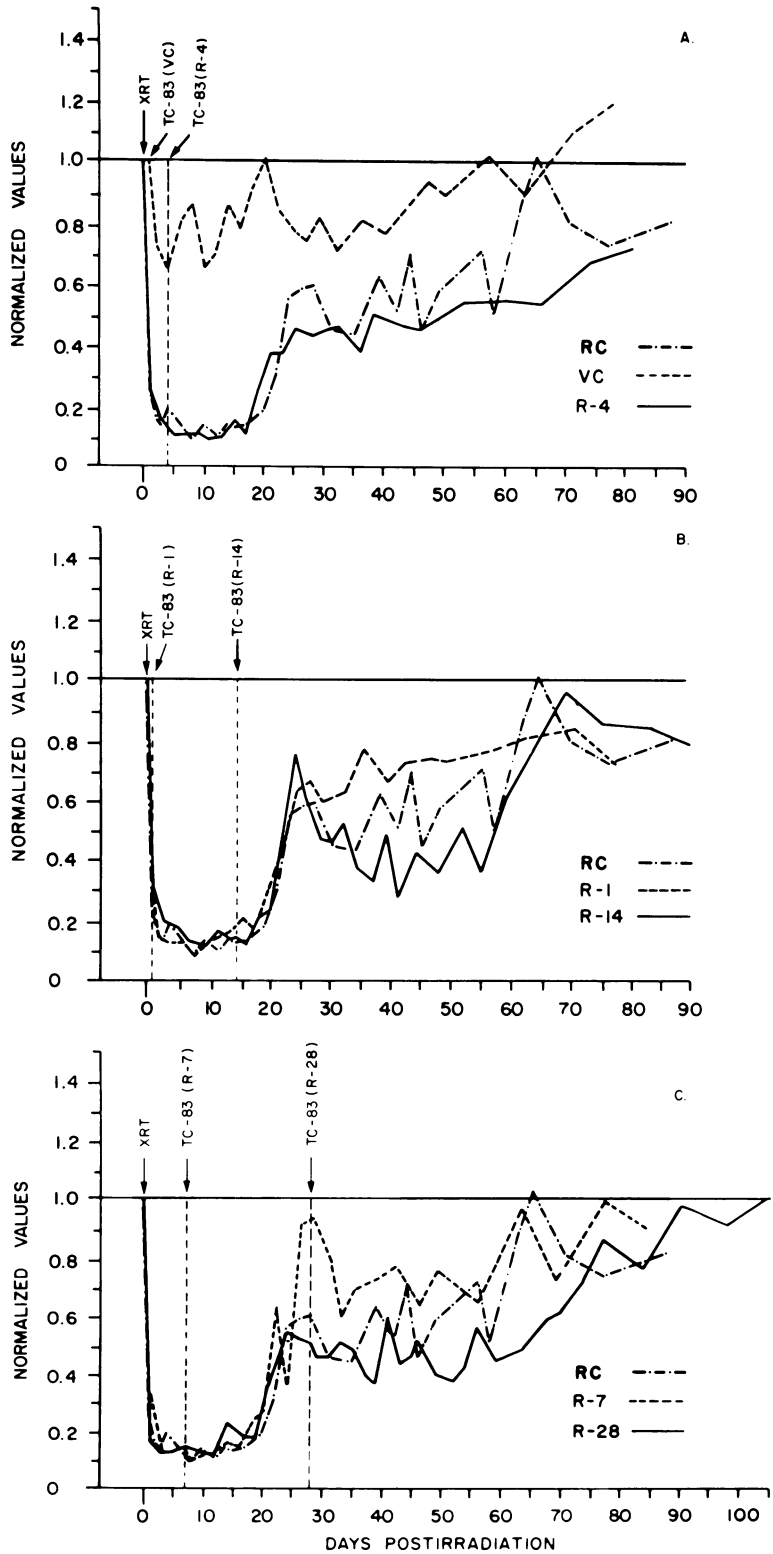

FIG. 1. *Normalized peripheral blood lymphocyte counts of rhesus monkeys (0.02 = a lymphocyte count which is 20% of the mean of five control values obtained on consecutive days from the same monkeys just before the study): RC, those exposed to 400 R to total-body X radiation, no TC-83; (A) VC, monkeys received TC-83, but no radiation; R-4, irradiated 4 days before TC-83 infection; (B) R-1, irradiated one day before TC-83 infection; R-14, irradiated 14 days before TC-83 infection; (C) R-7, irradiated 7 days before TC-83 infection; R-28, irradiated 28 days before TC-83 infection.*

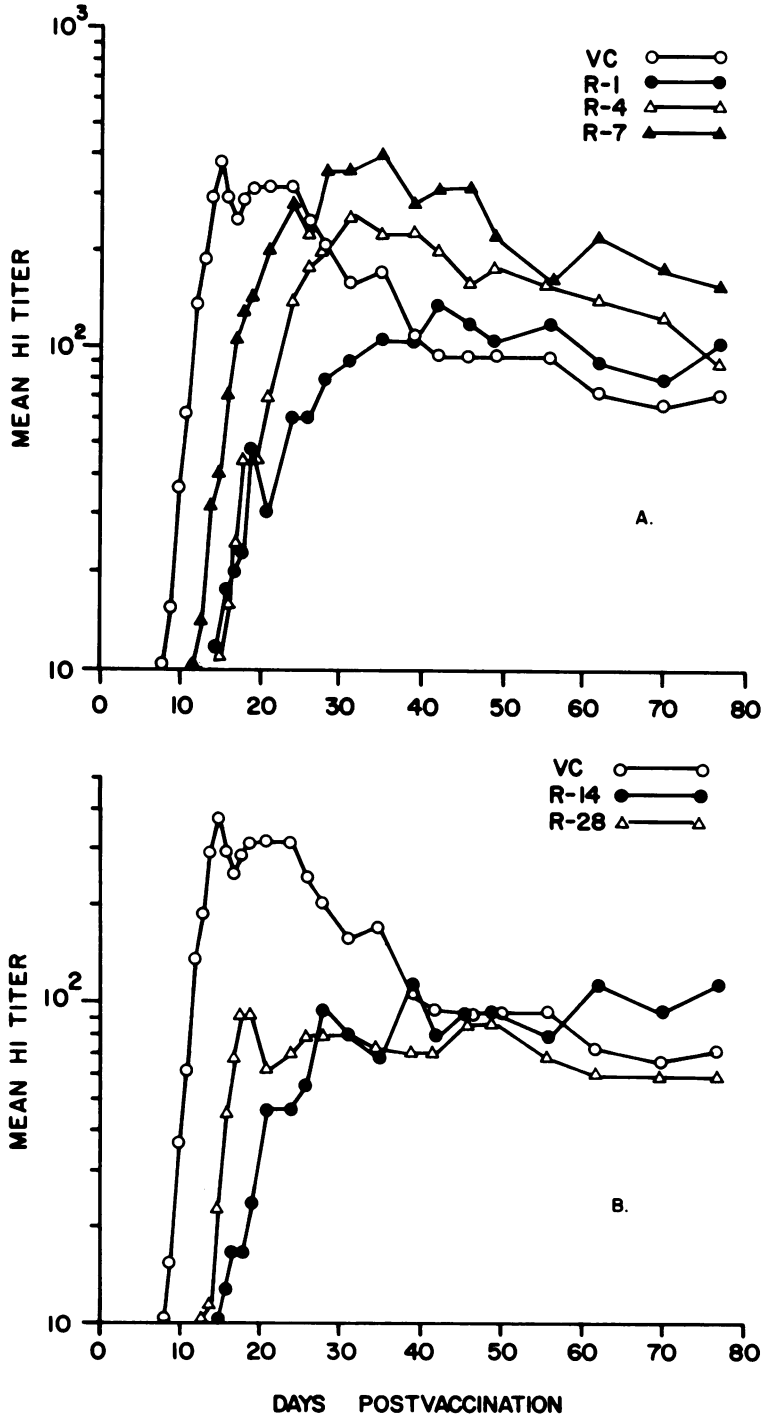

FIG. 2. *Geometric mean serum HI titers of rhesus monkeys given VEE virus, TC-83, by the subcutaneous route; VC, (O) TC-83-vaccinated control group, no radiation; (A) (●) those exposed to 400 R of total-body X radiation 1 day before TC-83 vaccination, (△) irradiated 4 days before TC-83 vaccination, (▲) irradiated 7 days before TC-83 vaccination; (B) (●) irradiated 14 days before TC-83 vaccination, (△) irradiated 28 days before TC-83 vaccination.*

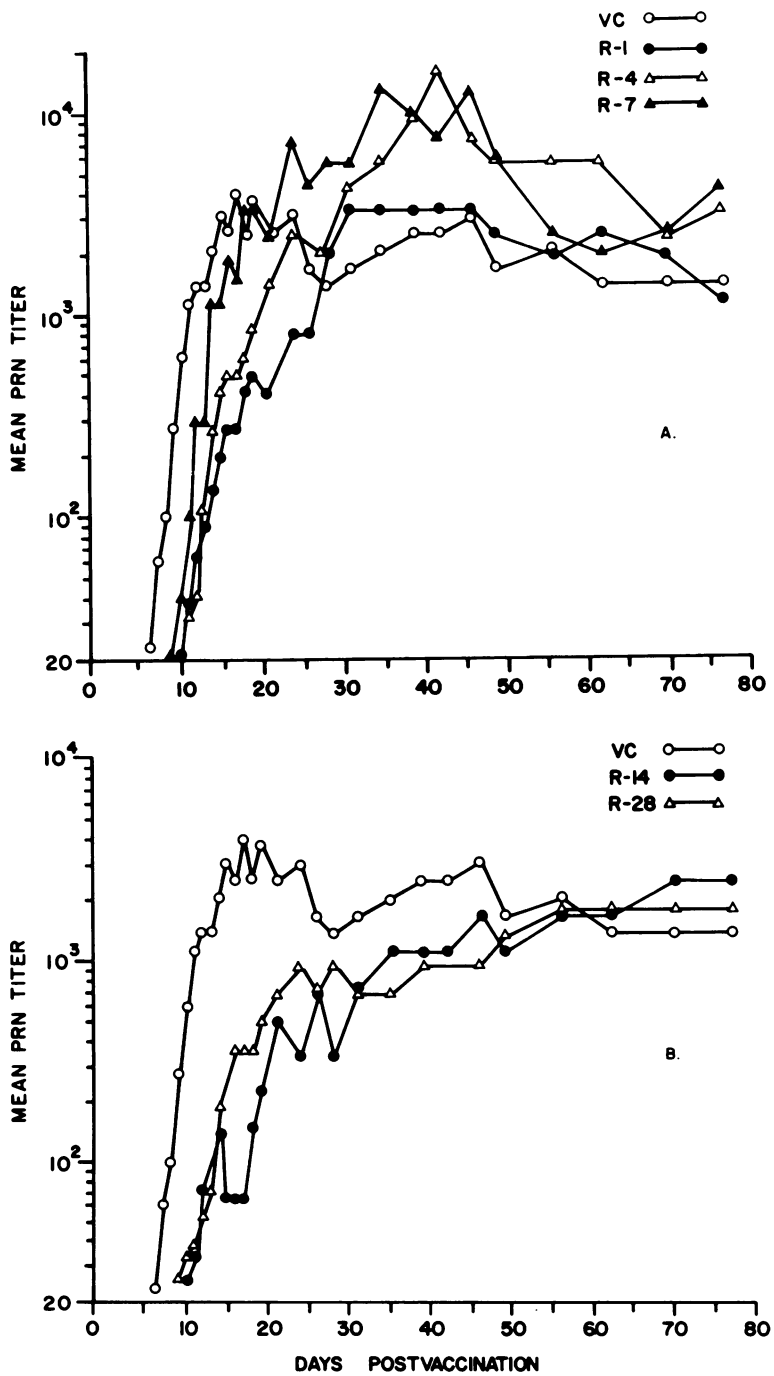

FIG. 3. *Geometric mean serum plaque reduction neutralization titers of rhesus monkeys given VEE virus, TC-83, by the subcutaneous route; VC, (○) TC-83-vaccinated control group, no radiation; (A) (●) those exposed to 400 R of total-body X radiation 1 day before TC-83 vaccination, (△) irradiated 4 days before TC-83 vaccination, (▲) irradiated 7 days before TC-83 vaccination; (B) (●) irradiated 14 days before TC-83 vaccination, (△) irradiated 28 days before TC-83 vaccination.*

the remaining monkeys in the group do not substantiate this assumption. Moreover, although irradiation or immunosuppressive drugs predispose animals to low-dose tolerance with relative ease (7, 17), development of immune tolerance with other viral immunogens has

invariably followed a viremic episode (6).

Persistence of viremia has been reported with other viruses in irradiated monkeys (31) and in immunosuppressed rodents (19); in the latter, an increase in tissue levels of virus was also observed. This deficiency in capability of irradiated animals to limit or suppress proliferation of viable antigen might result from suppression of antibody production, interferon production, or from delay in clearance by the RES (9, 26, 32). In the present studies, vaccinated-irradiated monkeys, unlike vaccinated controls, failed to develop measurable levels of interferon in their blood, suggesting that a deficiency of interferon may be a contributory factor. Initial clearance of inoculated virus by the RES, however, was essentially the same in both groups.

It is conceivable that depressed RES clearance is partly responsible for persistence of virus in the blood. Although it is reported that consequences of irradiation may either increase, decrease, or not change RES clearance in small nonprimate species (26), our preliminary TC-83 clearance studies in monkeys indicated that neither total body irradiation nor RES blockade with colloidal carbon delayed initial RES clearance of virus (12). Wagner et al. (33) found that vascular clearance of aggregated albumin in man was delayed in experimental viral infections; therefore, prolonged viremia in irradiated, TC-83-infected monkeys may still be due in part to delayed RES clearance of virus during infection.

The delay in the onset of detectable viremia in some irradiated monkeys suggest a deficient, susceptible, target-cell population and consequent slow development of detectable levels of viremia. Cells undergoing rapid cell division tend to be more radiosensitive, and VEE virus has known radiomimetic, particularly lympholytic, properties (2, 10). It could be postulated that, after repair of sublethal radiation injury and mitotic delay, there is a large, partially synchronized, population of host cells susceptible to viral replication, hence increased and prolonged virus levels, or possibly a secondary susceptible population of host cells.

Our findings indicate that radiation potentiates TC-83 infection in monkeys, as evidenced by increased serum titers of virus, but fails to enhance clinical signs of illness attributable to VEE. Evidence suggests that some fatalities in sublethally irradiated-vaccinated groups resulted from an increased susceptibility to pulmonary infections with secondary invaders. The surviving monkeys in the R-14 group showed a late decrease in their peripheral blood lymphocyte counts (Fig. 3B) when compared to the RC animals. Since TC-83 is responsible for some degree of lymphocytopenia in monkeys (Fig. 3A), we attribute the deaths of eight animals in the R-14 group to the radiomimetic stress of TC-83 superimposed on an already severely depressed hematopoietic capability at a critical time.

The initial delay in observable antibody in the irradiated monkeys correlates closely with the prolongation of viremia and magnitude of antibody response with antigenic mass (course of viremia) as well. The temporal studies clearly illustrate that, at a critical time after irradiation, the capability of the host to respond to a replicating antigen changes from a somewhat depressed state to relatively normal or enhanced activity. In contrast with nonreplicating antigens, enhancement of antibody has generally been reported when antigen injection precedes irradiation by a few hours (32), although Bogden et al. (4) found enhancement of immunological response in monkeys when irradiation preceded antigen injection.

It has been reported that a prolonged latent period in antibody development followed by a normal or enhanced antibody response is indicative of inhibition of the antibody-synthesizing mechanism, i.e., the proliferation and maturation of antibody-forming cells, without affecting induction (antigen recognition). In contrast, a decreased antibody response is interpreted as a depression of both induction and antibody synthesis mechanisms (4, 32). In this study, the HI antibody titers of monkeys in the R-1 group (Fig. 1A) were depressed and the latent period was prolonged; this could be interpreted as depression of both the induction- and antibody-synthesizing phases. Monkeys from the R-4 and R-7 groups showed near-normal or above-normal HI and serum-neutralizing titers, suggesting that the induction phase was not affected greatly, but that depression of the synthesizing mechanism may have been responsible for the prolonged latent period. This is consistent with interpretations of others in studies with a nonreplicating antigen in irradiated monkeys (4).

The induction phase may have been affected even more severely in our studies than is borne out by the data, because prolonged antigenic stimulation and/or exposure to increased antigenic mass through viral replication in the host probably contributed significantly to the HI and serum-neutralizing antibody responses. Other explanations for enhanced immune response in this situation may include depletion of T cells (thymus-dependent lymphocytes) that would normally exert a suppressive, regulatory influence on B-lymphocyte subpopulations (15).

Another effect by sublethal, total-body irradi-

ation on antibody synthesis other than proliferation and maturation was suggested by prolongation of the production period, i.e., the time from first appearance of newly formed antibody to peak titer, which resulted from a decrease in the rate of HI and serum-neutralizing antibody appearance. The delay in appearance of peak antibody titer in all irradiated-vaccinated monkeys is consistent with other reports on the response of irradiated monkeys (4, 23) and rodents (32). The depressed and delayed humoral antibody responses of animals in the R-14 and R-28 day groups may be related to the delay in repopulation of lymphopoietic tissues in these irradiated animals.

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