Effect of 5-Bromodeoxyuridine on the Hemolysin Response in Rabbits.* (31821)

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The initial hemolysin response following one intravenous injection of $10^{9.2}$ sheep red cells (sRBC) per kg rabbit after a latent period rises rapidly to peak titer and then declines slowly, as we reported previously(10-14).

Various procedures and materials, however, modify this sequence with respect to the length of the latent period, *i.e.*, length of time between injection of antigen and appearance of detectable antibody, rates of hemolysin rise, length of production to peak titer and peak titer. In all of this work, timing and dosage of the various reagents have been important(17). For example, on the one hand, small doses of X rays (25-100 R) do not alter hemolysin production in rabbits when the antigen is given 1 or 2 days afterwards, but stimulate it when the antigen is given 2 days or 2 hours beforehand, as manifested by high hemolysin peak titers attained at high rates after a latent period of normal length(11,15). On the other hand, large doses of X rays (400-700 R) significantly depress all parameters of the hemolysin response, *viz.*, decrease titers and rates and lengthen time intervals, when sRBC are given 1 or 2 days afterwards, whereas they enhance peak titer—although after a delay—when the antigen is given 2 days to 2 hours before the X rays(11-16). Furthermore, the X-ray-induced depression by 400 R can be neutralized by yeast and colchicine when given near or at the time of the antigen injection, as manifested by normal or above normal hemolysin levels—although after a delay. These materials also exert an adjuvant action on hemolysin production, *i.e.*, peak titers are significantly higher than those in unirradiated rabbits, as reported by Jaroslow and the senior author(3,4,9). These authors, from the foregoing and other data on nucleic acid digests (2,9), concluded that colchicine and other materials act by releasing nucleic acid degradation products directly or indirectly.

As an outcome of the foregoing studies and because of the work of Djordjevic and Szybalski(1), large and small doses of the nucleoside analogue, 5-bromodeoxyuridine (BUDR), were tested in the present report in unirradiated and irradiated rabbits given sheep red blood cells. Djordjevic and Szybalski studied the human sternal marrow cell line, D98S, *in vitro* and reported that such cells become highly susceptible to ultraviolet light and to X rays in the presence of 3×10^{-5} M BUDR which is incorporated into both strands of the DNA molecules of the cells in place of up to 45% of the natural component, thymidine. Since their work, Merritt and Johnson(6,7) and Johnson(5) tested the effect of the related analogue, 5-fluoro-2-deoxyuridine (FUDR), in unirradiated mice given bovine gamma globulin (BGG) or human gamma globulin (HGG). They found that FUDR acted as an adjuvant when injected within an hour of the time the antigen was injected and depressed the responses when BGG and HGG were injected before the analogue at 1-2 days and 1-4 days, respectively.

The present experiments indicate that BUDR acted as an adjuvant but did not depress the hemolysin response in unirradiated rabbits and did not modify X-ray-induced depression or enhancement.

Materials and methods. Young adult rabbits of the Dutch Belted variety purchased from Bunnyrun, La Puente, Calif., were fed on Rockland ration. In general, within a month of arrival, 20 rabbits, in groups of 4 or 5 weighing 2-2.5 kg, were tested under a variety of conditions with antigen and BUDR.

The antigen consisted of fresh sheep red cells (sRBC). A photometrically standardized suspension was injected intravenously at the rate of $10^{9.2}$ cells (approximately 1 ml

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TABLE I. Means ± Standard Errors for 5 Parameters of the Initial Hemolysin Response to Indicate the Effects of BUDR in Unirradiated and Irradiated Rabbits Injected Intravenously with $10^{9.2}$ sRBC per kg Rabbit.

Series*	Peak hemolysin log titer	Rate (k) of accumulation: First and most rapid	Rate (k) of accumulation: Average to peak titer	Length in days of: Latent period	Length in days of: Hemolysin rise to peak titer	No. of rabbits in sample†
		BUDR within an hour and a half of sRBC (No X)‡				
1 (L)	3.91 ± .09A	2.40 ± .16A	1.24 ± .21	2.88 ± .03	6.79 ± .61	11
2 (S)	3.77 ± .10B	1.91 ± .13	1.20 ± .19	2.68 ± .11	6.33 ± .64	15
		BUDR 2 days after sRBC (No X)				
3 (S)	3.32 ± .18	2.05 ± .17	1.01 ± .12	3.10 ± .18	5.78 ± .73	8
		sRBC alone (Control: no BUDR or X)				
4	3.48 ± .08	1.88 ± .09	1.10 ± .11	2.86 ± .13	6.41 ± .64	19
		BUDR + sRBC 1 day after 400 R				
5 (L)	1.75 ± .18	.33 ± .08	.24 ± .07	11.55 ± .89	11.07 ±1.48	12 (2)
6 (S)	2.09 ± .09	.46 ± .07	.24 ± .03	9.15 ± .58	12.22 ± .88	17
		sRBC 1 day after 400 R (Control showing X-ray-induced depression)				
7	1.89 ± .12	.40 ± .06	.22 ± .03	9.36 ± .52	11.34 ± .99	18 (3)
		BUDR + sRBC 1 day before 400 R				
8 (L)	4.01 ± .10	1.64 ± .10	.84 ± .07	3.33 ± .19	8.27 ± .43	18
9 (S)	3.99 ± .07	1.99 ± .19	.79 ± .04	2.93 ± .20	8.78 ± .30	8
		sRBC 1 day before 400 R (Control showing X-ray-induced enhancement)				
10	3.81 ± .10	1.86 ± .13	.80 ± .04	3.37 ± .14	8.52 ± .49	15

* BUDR was given intraperitoneally as a total large (L) amount of 200 or 400 mg per kg to series 1, 5 and 8 or as a total small (S) amount of 50 mg per kg to series 2, 3, 6 and 9.

† The negative hemolysin responses of the rabbits listed in parentheses are included in the means of peak titer only where they are given an arbitrary log value of 1.

‡ Values for P for differences in series 1, 2 and 3 from control series 4 are indicated as follows: A, 0.002-0.008; B, 0.04; other differences >0.05.

of a 10% suspension) per kg rabbit. This single dose of antigen leads to the production of the anti-Forssman immune gamma 1 (γM or IgM) hemolysin. More intensive immunization than this single dose is needed to elicit the anti-Forssman gamma 2 (γG or IgG) hemolysin(8).

Beginning at the time that the anitgen was injected (day 0), about 2 ml of blood were obtained from the marginal ear vein of each rabbit 4 times for the first week, 3 times a week for the following 3 weeks and sometimes twice a week for an additional 2 weeks. The sera from these bleedings were titrated to obtain a photometric 50% hemolysis endpoint according to our method(10-16). The resulting hemolysin titers from each rabbit were plotted semilogarithmically against time to obtain means ± standard errors for various parameters, such as the latent period, rate and length of hemolysin production to peak titer and peak log titer (Table I). When hemolysin rose to peak titer rapidly at first and then more slowly, an average rate of rise was calculated from a line drawn from a log titer of 1 at the time hemolysin was first detected to peak log titer at the time it was attained. Statistical analyses of the means of the parameters, involving Student's t test, were made on peak log titers and on arithmetic values for other parameters(11).

For total body irradiation, rabbits were immobilized in small perforated aluminum boxes. The boxes were turned at the midpoint of irradiation. The horizontal beam was delivered at 39 to 40 R per minute through 0.5 mm Cu and 1 mm Al filters (half-layer value = 1.70 mm Cu) at a target distance of 65 cm from the center of the rabbit by a machine operating at 250 kv and 15 ma.

The analogue, 5-bromodeoxyuridine (BUDR), obtained from the California Corporation for Medical Research, Los Angeles, Calif., was always injected intraperitoneally through a clean shaven area of the abdomen

via a 19 gauge needle in a dose per kg rabbit. As it was difficult to dissolve, a requisite amount in a 50 ml centrifuge tube was shaken in a heated solution containing 0.85% sodium chloride and 0.1 *N* hydrochloric acid and was injected after sufficient cooling. In series 1, 5, and 8, a large amount of 100 mg per kg rabbit per 10 ml of diluent was given either 2 times, *i.e.,* 45 minutes before and 45 minutes after injection of antigen, or 4 times, *i.e.,* immediately after antigen injection and at half hourly intervals thereafter. The results from these two treatments were indistinguishable. In series 2, 3, 6 and 9, small amounts were given. Thus, 50 mg per kg rabbit in 5 ml of diluent was given once one-half hour before the injection of antigen or half that amount was given twice, *i.e.,* 45 minutes before and 45 minutes after the injection of antigen.

Experimental results. BUDR was not toxic when given intraperitoneally in 100 mg doses per kg 2 or 4 times within an hour and a half interval to young healthy rabbits of either sex weighing between 2.0 and 2.5 kg and did not add to the injurious effect of 400 R total body irradiation. Two doses of 100 mg per kg, however, were toxic to rabbits weighing 2.6 kg or more (5 of 7 died), and one large dose of 300 or 600 mg per kg was not tolerated well by rabbits weighing less than 2.5 kg (4 of 7 died).

Table I gives means ± standard errors for 5 parameters of the hemolysin response following the injection of sRBC into 7 series of rabbits given BUDR and into 3 control series not given the compound. The analogue was tested for 4 possible effects. It was tested for its adjuvant action in unirradiated rabbits when given within an hour and a half of the antigen (series 1 and 2 *vs* control unirradiated series 4), for its effect on the latent period in unirradiated rabbits when given 2 days after the antigen (series 3 *vs* control unirradiated series 4), for its restorative effect of X-ray-induced depression when it and the antigen were given one day *after* 400 R (series 5 and 6 *vs* control series 7) and for its effect on X-ray-induced enhancement when it and the antigen were given one day *before* 400 R (series 8 and 9 *vs* control series 10). Large as well as small amounts were given to test 3 of the 4 possibilities. We obtained the following results.

(1) Large doses when given at the time sRBC were injected into unirradiated rabbits exerted an adjuvant action on hemolysin production. Thus, 2 of the 5 parameters in series 1, *i.e.,* peak titer and the initial rate of rise, were significantly higher than those of control series 4. Small doses (series 2) were also effective in that peak titer in series 2 was significantly higher than that in series 4.

(2) Small doses when given 2 days after sRBC to unirradiated rabbits did not appreciably modify the ensuing response. None of the 5 parameters in series 3 was significantly different statistically from those in control series 4.

(3) Neither large nor small doses when given with the antigen a day *after* 400 R exerted any restoration of hemolysin production. All 5 parameters of the mean responses in irradiated series 5 and 6 were as depressed as were those in control irradiated series 7 and were significantly depressed ($P<0.001$) as compared to control unirradiated series 4. This depression, as compared to the unirradiated controls and as noted previously, consisted of marked decreases in peak titer and rate, and marked increases in the length of the latent period and hemolysin rise to peak titer.

(4) Finally, neither large nor small doses when given with the antigen a day *before* 400 R reduced or increased hemolysin production. The 5 parameters of the mean responses in irradiated series 8 and 9 were not significantly different statistically from those in irradiated control series 10. Moreover, in series 8, 9 and 10, as compared to control series 4, the higher peak titers ($P = 0.002-<0.001$) were reached after a longer total rise ($P = 0.03-0.002$) during which the average rate of rise was slower ($P = 0.05-0.02$). Thus, the excess amount of hemolysin produced in rabbits given antigen 1 day before 400 R was not modified by BUDR.

Discussion. We expected that BUDR, as compared to suitable controls, would make hemolysin synthesis more sensitive to radia-

tion and might also increase hemolysin production in rabbits irradiated at a critical time because it was reported(1) to have made cells highly susceptible to irradiation. Neither of these results was realized. In the irradiated series, the analogue did not further depress hemolysin formation (*cf* series 5 and 6 with 7), it was not restorative (*cf* series 5 and 6 with 4), and it did not modify enhanced formation (*cf* series 8 and 9 with 10). That the analogue did not either inhibit or increase the X-ray-induced effect when given before irradiation falls in line with its failure to depress or to restore X-ray-induced depression when given after irradiation. In the unirradiated series, however, BUDR produced an unequivocal adjuvant effect on the hemolysin response (*cf* series 1 and 2 with 4). This effect was primarily associated with induction of antibody formation because more hemolysin was formed at a faster rate during a normal length of time. It was not associated with any appreciably faster development of the antibody-synthesizing mechanism because the latent period was normal in length.

BUDR thus differed from colchicine and yeast which produced both restorative and adjuvant effects on hemolysin production in irradiated and unirradiated rabbits, respectively(3,4). BUDR also differed in most respects from FUDR as tested on anti-BGG and anti-HGG responses in unirradiated mice by Merritt and Johnson(6,7) and Johnson (5). Thus, the adjuvant effect of FUDR was not only characterized by a higher peak titer (which we obtained in series 1 and 2) but by a shorter latent period than in controls, and FUDR exerted a depressing effect (which we did not find in series 3), as evidenced by low peak titers and a longer latent period when the antigen was injected 2 days beforehand. Some of these differences would no doubt disappear if the same experimental model had been used to test each of the analogues because specific irradiation exposure at specific times affect the hemolysin response in the rabbit differently from that in the mouse(17).

We had come to believe that an agent exerting a restorative effect on antibody-forming capacity in irradiated animals would also exert an adjuvant effect in unirradiated animals, as demonstrated by colchicine and yeast (3,4), and this relationship probably holds qualitatively. In the present experiments, however, BUDR only acted as an adjuvant. A suitable interpretation of the results will have to await further studies, but relative cytotoxicity may play a role. Thus, on the one hand, the dual action of colchicine was explained in part by its toxicity which probably releases nucleic acid degradation products(2, 9). BUDR, on the other hand, can be tolerated in relatively large amounts by rabbits.

Summary. The analogue, 5-bromodeoxyuridine, when injected intraperitoneally within an hour and a half of an intravenous injection of sheep red blood cells, produced an unequivocal adjuvant effect on the hemolysin response in unirradiated rabbits as gauged by high peak titer. It was, however, ineffective when injected 2 days after the sheep red cells into unirradiated rabbits and did not modify X-ray-induced enhancement or depression when it and the antigen were injected one day before and one day after 400 R, respectively.

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Malignant Transformation and Maturation in Non-Dividing Cells During Polymer Tumorigenesis.* (31822)

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Many theories of carcinogenesis have a common denominator: the concept that development towards malignancy can be regarded as an evolutionary process which stretches over a chain of premalignant cell generations gradually selecting for more autonomous clones. A contradiction is provided by certain viral tumors. Here, infection of a cell with the virus virtually coincides with complete malignant transformation. However, so far this mechanism is being considered exceptional rather than generally compatible with other forms of carcinogenesis(1).

Investigations have been carried out in this laboratory on tumorigenesis following subcutaneous implantation of plastic film pieces into mice. Results already published(2) have changed previous views regarding the site of carcinogenic development in that the premalignant cells were exclusively found to be firmly attached to the plastic film inserts until shortly before tumors become palpable. Experiments in this new direction have yielded data which strongly suggest that the carcinogenic events take place in a non-dividing cell population remaining stationary throughout the premalignant phase.

Experiments and results. The design of the experiment was similar to that described previously(2). Inbred mice (CBA-T_6 carrying the T_6-marker chromosomes) at the age of 1½ to 2 months received in both flanks subcutaneous inserts of vinyl chloride acetate-coverslips ("dispo-slips"), 15 × 22 mm in size. Inserts and tissue capsules (which surrounded the inserts as a result of a foreign body reaction) were cut in thirds after time intervals of from 2 weeks to 16 months. One portion was left in the original animal. A second portion was used for cultural, karyological, and/or histological examinations. The plastic and capsule pieces of the third portion were carefully separated and then individually transplanted into CBA-H recipient mice (which are syngeneic with CBA-T_6 mice but do not carry T_6-chromosomes). Tumors, with specific T_6-chromosomes, appeared simultaneously up to 8 months later in the original and the corresponding recipient animals if transplantation was carried out 6 or more months after initial insertion of the plastic films. If the latent period (*i.e.,* from time of transplantation until tumor appearance) was observed to last longer than 4 weeks, the tumors developed from transplanted film pieces only, not from capsule tissue. These results were in agreement with those described before(2). Evidently, the carcinogenic events take place among cells residing in multiple foci on the plastic film but not in the surrounding tissue as was claimed in the earlier literature (recently reviewed by Bischoff(3)).

As outlined in the foregoing, plastic film fragments were put aside from each single transfer experiment. Accordingly, these fragments were known to carry cell populations with exactly determined degrees of premalignant maturation in terms of time elapsing until tumor appearance. The film fragments were treated in various ways. They were stained immediately after removal from the original carrier animal with Pappenheim's combined May-Gruenwald-Giemsa procedure or with a combined Tetrazolium-Feulgen procedure developed by us for this purpose(4).

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