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STUDIES ON IMMUNOSUPPRESSION BY PURINE NUCLEOSIDE ANALOGUES—I

EFFECTS ON THE IMMUNE RESPONSE TO SHEEP RED BLOOD CELLS IN MICE*

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Abstract—The time dependence of optimal immunosuppressive activity of several purine nucleoside analogs was studied using the Jerne *et al.* plaque assay technique to§ enumerate cells forming antibody to sheep red blood cells (SRBC) in mouse spleens. A single dose of drug was given before or at different times after a single dose of SRBC in order to determine the drug-sensitive phase of anti-SRBC antibody production in mouse spleens. α-2′-Deoxythioguanosine exhibited maximum activity during the proliferative phase of the immune response. β-D-Arabinofuranosyl-6-mercaptopurine showed activity only during a limited period of the proliferative phase. β-D-Ribosyl-6-methylthiopurine was active throughout the response. The periodate oxidation product of β-D-ribosyl-6-methylthiopurine (MMPR-OP) was active when given either before or after SRBC, as was the periodate oxidation product of inosine. 6-Methylthiopurine, one of the cleavage products of MMPR-OP was active only if given after SRBC. The results are discussed in terms of the effects of these drugs on cellular kinetics and metabolism.

PURINE nucleoside analogs have been introduced in cancer chemotherapy mainly to overcome drug resistance to certain purine analogs.[1, 2] The structural alterations in these "fraudulent" nucleosides result in activity on a more restricted target area of cellular metabolism. Several of the purine nucleoside analogs previously studied as carcinostatic compounds have been identified as immunosuppressive agents. Some of them (e.g. Ara-6-MP‖ and MMPR-OP) inhibit the allograft response in a system using tail-skin transplantation between two different inbred mouse strains, but do not seem to suppress the humoral response under the same conditions as measured by hemagglutination in mice immunized against SRBC.[3–6]

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§ N. K. Jerne and A. A. Nordin, *Science, N.Y.* **140**, 405 (1963). N. K. Jerne, A. A. Nordin and C. Henry, in *Cell-Bound Antibodies* (Eds. B. Amos and H. Koprowski), p. 109. Wistar Institute Press, Philadelphia (1963).

‖ Abbreviations used: Ara-6-MP, β-D-arabinosyl-6-mercaptopurine; MMPR, β-D-ribosyl-6-methylthiopurine; MMPR-OP, the periodate oxidation product of MMPR; SRBC, sheep red blood cells; PFC, plaque-forming cells; I-OP, the periodate oxidation product of inosine; α-TGdR, α-2′-deoxythioguanosine; MMP, 6-methylthiopurine.

In the present study, employing sheep erythrocytes and the Jerne *et al.* plaque technique,[7, 8] we wanted to obtain further information about the interaction of these purine nucleosides with the cellular kinetics of the developing immune response to SRBC in mice. The plaque technique allows a precise analysis of the early phase when 19S antibody levels in the serum are far below the sensitivity of conventional serological methods. In addition, changes in the number of antibody forming cells are revealed almost immediately, but such changes only become apparent in serum antibody titers after considerable delay.[9] In order to simplify the study of the time dependence of effective immunosuppression, a single dose of drug was injected at certain times relative to a single injection of antigen. Of course, such a treatment does not completely define the effectiveness of a drug from a clinical point of view. On the other hand, accumulative treatment makes it much harder to reach conclusions about mechanisms of drug action. Several doses at a maximum tolerated level (fraction of LD_{10}) of MMPR and α-TGdR are effective.[3, 4] In contrast, multiple sublethal doses of Ara-6-MP[3, 4] and near toxic or toxic doses (LD_{50}–LD_{70}) of MMPR-OP[5] did not inhibit circulating antibody production.[5] An attempt was made to correlate distribution, metabolism and metabolic effects with immunosuppressive capacities.

MATERIALS AND METHODS

Determination of 19S-hemolysin-producing cells. Male CD-1 random-bred mice (Charles River Mouse Farms, Wilmington, Mass.) were used in all experiments. They were injected intraperitoneally (i.p.) with 0·2 ml of a 10% (v/v) solution of SRBC (Colorado Serum Company, Denver, Colo.) in isotonic saline. The number of hemolysin-producing cells (PFC) was measured according to a slightly modified technique of the direct hemolytic plaque test described by Berenbaum.[9] In order to prepare spleen cell suspensions, the spleens were removed and cut into small fragments in cold Medium 199 (Hyland Laboratories, Los Angeles, Calif.), and the cells were teased out by applying gentle pressure. Particulate matter was removed from cell suspension by filtering it through glass wool. The wisp of glass wool we used for this purpose and the fast passage of the cell suspension did not lead to significant adsorption of PFC during this procedure. The volumes of the suspensions were adjusted to contain the desired number of spleen cells for plating. In the initial phase of the response (day 0 to 2), one spleen was suspended in a final volume of 0·4 ml and 0·2 ml was plated. In the proliferative phase (day 3 to 5), 10 ml of Medium 199 was added and 0·05 ml of the suspension was plated. Finally, in later phases of the response (day 6 to 10), 0·4 ml of the suspension and from day 12 on, 2·0 ml of the suspension was plated. After 12-hr storage in the refrigerator, the unstained plates were examined with a stereo dissection microscope, using a 7·5× magnification. The total number of plaques per individual spleen was calculated by multiplying the number of PFC per plate by the dilution factor. For each group of drug-treated mice, there was 1 control group. The averages of counts from five mice per day per experimental group, expressed as PFC counts per whole spleen, were used to draw the response curves. The observations covered a 16-day period.

Preparation of I-OP. The oxidation of inosine with periodic acid was carried out under conditions identical to those used for the synthesis of MMPR-OP,[5] to afford a colorless, amorphous product. A lyophilized sample of I-OP was submitted for analysis:

Anal. Calc. for $C_{10}H_{10}N_4O_5 \cdot 1{\cdot}5\ H_2O$(293·2): C, 40·96; H, 4·47; N, 19·11. Found: C, 40·70; H, 4·75; m, 18·89.

Paper chromatographic analyses similar to those carried out on MMPR-OP[10] showed that the sample contained none of the starting material and little or no free hypozanthine.

Drug treatment. All drugs were injected i.p. in a volume of 0·25 ml. α-TGdR was dissolved in isotonic saline by adding one equivalent of NaOH; the final pH was about 8. A single dose of 80 mg/kg was injected.

MMPR-OP was partially dissolved in absolute ethanol; saline was then added to give a solution of 4% (v/v) ethanol. A single dose of 100 mg/kg was injected.

Ara-6-MP was dissolved in a solution containing 0·13 M NaCl and 0·02 M $NaHCO_3$, pH 7·4. Under these conditions, 10 mg/ml can be dissolved at 37°.* The drug was used at a dose level of 500 mg/kg, given in two divided doses 8 hr apart.

All the other drugs were dissolved with vigorous stirring in isotonic saline (pH adjusted with NaOH to 7·4). They were given in the following amounts: MMPR, 70 mg/kg; I-OP, 96 mg/kg (equimolar to MMPR-OP); MMP, 50 mg/kg.

The purpose of this investigation was to compare the time dependence of effectiveness with known biochemical effects rather than determine maximum effectiveness. Therefore comparative toxicity studies, to allow use of equitoxic doses, have not been done. In general, the highest nontoxic dose, corresponding to a fraction of LD_{10}, was chosen. Data on the toxicity of α-TGdR,[3] MMPR,[5] MMPR-OP,[5] MMP[11] and Ara-6-MP[3, 12] have been published previously. One dose of Inosine-OP at 200 mg/kg produced no external signs of toxicity in C3H mice.†

Mice were injected with SRBC on day 0 and each group was treated with a single dose of drug before or after antigen injection, as indicated. Control mice were injected with antigen only. Groups of mice were killed at intervals after antigenic stimulation, their spleens were removed, and the number of 19S-PFC were determined.

Statistical analysis. Differences between individual groups, as indicated in Figs. 1 and 2, were assessed by the method of Hogben.[13]

RESULTS

Only 19S-(IgM) hemolysins were measured. Spleens of untreated control mice contained about 30 to 70 PFC. After stimulation with SRBC, the number of PFC did not substantially increase during the first day or two, the inductive phase. From day 1 or 2 to day 4 the proliferative phase, a rapid increase in PFC was observed, the peak being 5×10^5 to 10^6 PFC per spleen. From day 4 to 8, the productive phase, there was a more or less rapid fall in the number of PFC, varying from one experimental group to the other. Finally, the response leveled off at 10^3 to 10^4 PFC during the remainder of the experiment.

α-TGdR, as well as MMPR, had to be given either at the same time as or after the antigen injection in order to suppress the number of PFC (Fig. 1). α-TGdR was most effective if given at day + 2 or + 3 (Fig. 1, c and d), at which time the number of PFC was immediately depressed to about 1·8 per cent of the corresponding control value. If α-TGdR was given at day 0 or + 1, a decrease in the number of PFC was

* G. A. Le Page, unpublished experiment.
† J. P. Bell, unpublished observations.

not observed until day 3 (Fig. 1, a and b). A return to control values was observed only for the day 0 treatment. In contrast, MMPR given at day 0 or + 1 caused immediate suppression of the number of PFC to about 2·5 per cent of the control (Fig. 1, g and h). If MMPR was injected at day + 3, the effect was much smaller than that for α-TGdR on the same schedule (Compare Fig. 1, d and i). Administration of MMPR at day + 8 resulted in a transitory depression (Fig. 1, j). In general, recovery from MMPR treatment was found to be very rapid compared to that obtained with α-TGdR. MMPR-OP showed suppressive activity that was different from that of MMPR, especially in the early phase of the response to SRBC. MMPR-OP was effective when given 3 days before the antigen (Fig. 2, a). Apparently the oxidized sugar moiety of MMPR-OP is responsible for this particular result. To test for this, mice were treated with I-OP. Since inosine is a normal constituent of purine metabolism, any effect observed with I-OP has to be ascribed to the oxidized ribose moiety. Indeed, the results demonstrated a similar pattern of immunosuppression between MMPR-OP and I-OP (Fig. 2, a and f). Pretreatment with MMPR-OP or

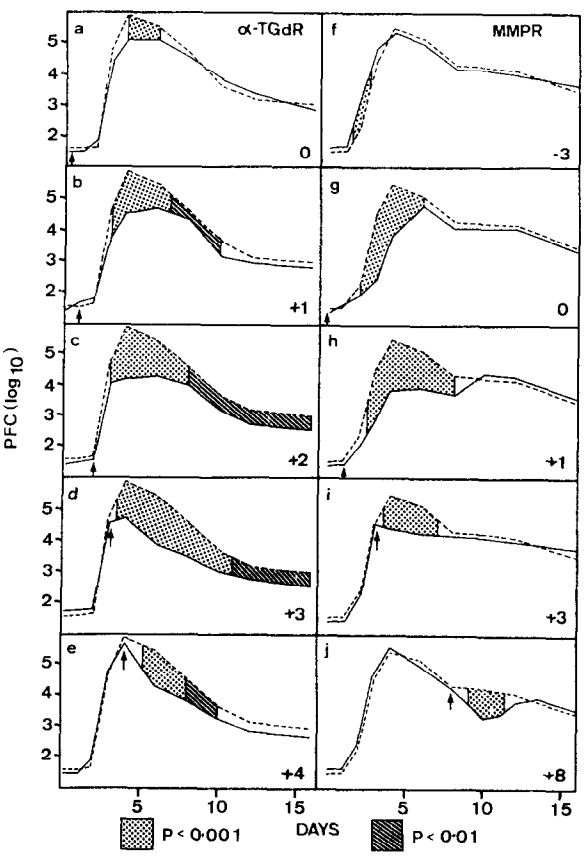

FIG. 1. Effects of α-TGdR (a to e) and MMPR (f to j) on hemolysin-forming cells. Every point on the curve represents an average of five mice. Sheep red blood cells were injected at day 0 and each drug was given on the days indicated in the right lower corner of each box and by the arrows. – – – –, control mice; ——, drug-treated mice.

I-OP led to a biphasic impairment of normal PFC development in that those periods both before and after the proliferative phase were more sensitive. Treatment with MMPR-OP 3 days before antigen seemed to be more effective than treatment 3-hr before antigen (Fig. 2, a and b). Administration of the drug on day 0 or + 1 did not significantly impair the proliferative phase, but had an effect on the productive phase (Fig. 2, b and c). If MMPR-OP was given during maximal accelerated production of hemolysin-forming cells (day 3), an immediate and pronounced suppressive effect was obtained. Since there is good evidence that MMPR-OP breaks down in a complex way, and since one of its metabolites is MMP,[10] the latter compound was tested in our system. Injection of MMP 3 days before antigen has no effect on the numbers of PFC, but resulted in an earlier onset of the proliferative phase (Fig. 2, h). The same result was obtained with MMPR (Fig. 1, f). On the other hand, MMP depressed the number of PFC when given at day + 3. The effect was appreciable if we consider that the dose of MMP used was only half that of MMPR-OP. Nevertheless, this dosage of MMP is much higher than the amount that can be expected from cleavage of MMPR-OP *in vivo*.[10] Ara-6-MP given on days − 3, 0 + 1, and + 2 had no effect on normal cellular kinetics (data not shown). If injected on day + 3, there was a delayed reduction of the number of PFC to about 3 per cent of the control values (Fig. 2, j). It

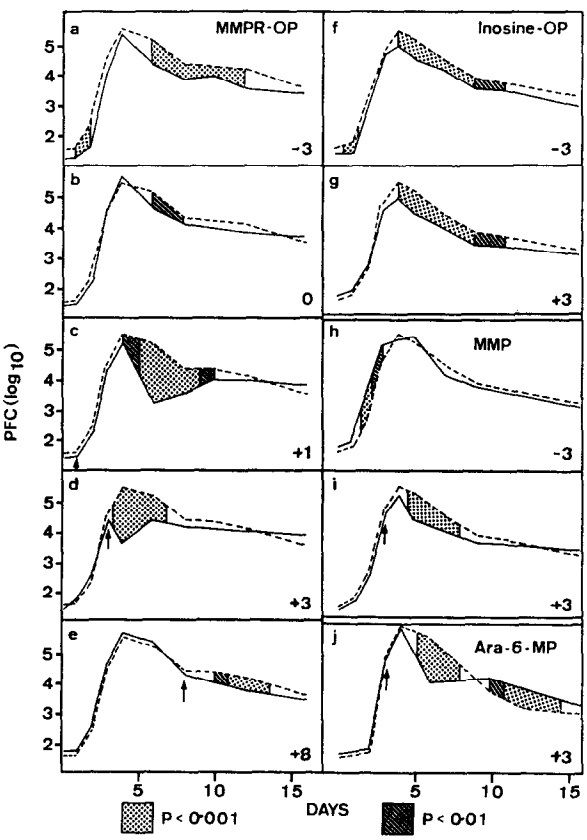

FIG. 2. Effects of MMPR-OP (a to e), I-OP (f, g), MMP (h, i) and Ara-6-MP (j). Other information is given in the legend to Fig. 1.

should be noted, however, that during the late productive phase, values up to five times higher than the controls were found in those groups given Ara-6-MP on day + 3.

DISCUSSION

The theoretical aspects of cellular proliferation in the immune response have been reviewed.[14] It seems that antigen stimulates proliferation as well as antibody synthesis among antigen sensitive cells.[15, 16] Nevertheless, there may be a distinct difference between the rate of appearance of the antibody forming function in cells and their rate of growth.[17-19] We base our discussion on a model, wherein transformation to an antibody producing cell without proliferation does not occur. As expected, the immunosuppressive activity of the nucleoside analogs studied was dependent on the timing of drug and antigen administration. These immunosuppressive effects appear to be related to the overall stage of differentiation and proliferation of the heterogenous spleen cell population at the time of treatment.

MMPR and MMP had no suppressive effect when given on day −3. Instead, the proliferative phase started earlier indicating that, as cells escaped the effects of these drugs, a homeostatic mechanism may have triggered an early proliferation among precursor cells.[9] Ara-6-MP had a similar effect after recovery from drug treatment. This point will be discussed in another paper.[20] The main difference between α-TGdR and MMPR was that MMPR was most active when given with the antigen or during the inductive phase, whereas α-TGdR was most active when given during the proliferative phase. The recovery after treatment was also faster for MMPR than for α-TGdR. There is good correlation between these results and the known metabolic effects of these drugs.

MMPR is phosphorylated by adenosine kinase in a large spectrum of mammalian tissues,[21] and the resulting nucleotide blocks *de novo* purine synthesis by feedback inhibition.[22, 23] MMPR does not appear to be incorporated into DNA.[22] A single dose of MMPR inhibited *de novo* purine synthesis in the spleens of normal mice for up to 24 hr.[23] Nevertheless, the recovery of antibody production in the spleens of our immunized mice was rapid, which may reflect, in part, the utilization of purine salvage pathways to overcome the blockade. The fact that MMPR was ineffective when given on day − 3 indicates that its selective concentration in those cells that phosphorylate it[10, 21] was not long-lasting. MMPR is not cleaved to MMP, but phosphatases convert the nucleotide back to MMPR, which is excreted largely unchanged in the urine.[10, 21]

α-TGdR is phosphorylated and a small amount is incorporated into mouse tumor DNA as a terminal nucleoside.[24, 25] The remaining α-TGdR, some thioguanine, and other metabolites are excreted in the urine relatively rapidly (50 per cent after 30 min*). Therefore it was not surprising that α-TGdR was most effective when given slightly before acceleration of DNA synthesis during the proliferative phase. MMPR may block RNA synthesis as well and, as a result, was active during the inductive phase during which accelerated RNA synthesis has been reported.[26] Recovery from the effects of α-TGdR is slower because its disruption of DNA structure can only be overcome by cellular repair processes.

Ara-6-MP was ineffective if injected before the proliferative phase. It showed

* G. A. Le Page and M. J. Wilson, unpublished experiments.

immunosuppressive activity only during the proliferative phase, which correlates with its partial blockade of DNA synthesis through inhibition of cytidine diphosphate reductase.[27] Since Ara-6-MP is neither cleaved[28] nor converted to nucleotide,[29] it is rapidly excreted in the urine (76 per cent after 6 hr).[28] Thus, Ara-6-MP is most effective only when injected at the peak of spleen cell proliferation.

The situation is more complex for MMPR-OP since several of its breakdown products may be active. Forty-six per cent of the injected dose is excreted in the urine after 1 hr. Only 1 per cent of the dose per gram of tissue is found 3 hr after injection but this low level is maintained for at least 4 days due to the unusual binding capacity of MMPR-OP.[10] Either the long-range effects of the original dose or the immediate effects of this bound residue might account for the long-term activity of MMPR-OP given on day − 3. Since we did not determine the total number of nucleated cells per spleen, we do not know to what extent toxicity towards precursor cells could be responsible for this effect. Evidence that part of this long-range activity of MMPR-OP is due to its oxidized sugar is found in the similar pattern of immunosuppressive activity of I-OP. The activity of MMP, one of the breakdown products of MMPR-OP, indicates that it contributes to the overall effect of MMPR-OP. A degradation product of MMPR-OP which we did not study, is glyoxal, a reactive cross-linking agent and antimetabolite (cf. discussion in ref. 10). MMPR-OP has been shown to block protein, RNA and DNA synthesis in mouse ascites tumor cells;[5] specific blockades were found in thymidine metabolism.[30] These metabolic blockades could explain the results obtained here.

The suppression of 19S antibody-forming cells observed for Ara-6-MP and MMPR-OP had no consequence on circulating antibodies, as measured by hemagglutinin titers.[4, 5] Even when MMPR-OP was given repeatedly, the hemagglutinin titers were the same as those of nontreated controls.* The lack of effect of Ara-6-MP on hemagglutinin titers, when given at 60 mg/kg/day,[4] may be due to the restricted period of optimal suppression of antibody-forming cells; no explanation is available in the case of MMPR-OP. However, the original explanation for the lack of inhibition of the hemagglutinin response by Ara-6-MP, that cells producing humoral antibody are spared,[6] does not hold for the higher doses used in the present study.

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