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Stimulatory Effects of 5-Iodo-2'-deoxyuridine on Number and Function of Splenic B- and T-Cells and of Macrophages in C3HeB/FeJ Mice[1]

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SUMMARY

5-Iodo-2'-deoxyuridine (IUdR) significantly increases the total number of spleen cells on the fourth day after administration of 100 mg/kg in normal C3HeB/FeJ male mice. The number of splenic B-cells [cells sensitive to the cytotoxic activity of a rabbit antiserum to mouse $F(ab)_2$, plus complement] increases on the same day. The number of T-cells (cells sensitive to the cytotoxic activity of a rabbit antiserum to mouse brain-associated θ antigen, plus complement) and of macrophages (adherent-phagocytic cells) does not increase.

Spleen cells from IUdR-treated mice show heightened responsiveness *in vitro* to *Escherichia coli* lipopolysaccharide or concanavalin A on Day 4 and Days 5 to 6, respectively. The rate of clearance of i.v.-injected colloidal carbon is also increased for 3 days after IUdR. Thus, IUdR is able to functionally activate either T- or B-cells or macrophages, but only to increase the number of splenic B-cells.

INTRODUCTION

IUdR[4] is now used in the treatment of certain DNA virus infections but may also find eventual use as an antineoplastic agent. In contrast to the immunosuppressive activity generally shown by antimetabolites, IUdR is able to stimulate antibody production to sheep RBC in mice (5). Because B-cells, T-cells, and macrophages participate in this immune response, it is possible that IUdR stimulates 1 or all of these cell types.

MATERIALS AND METHODS

Mice

C3HeB/FeJ male mice, 6 to 10 weeks old, were obtained from The Jackson Laboratories, Bar Harbor, Maine.

IUdR Treatment

IUdR (Calbiochem, Los Angeles, Calif.) was administered i.p. in 0.9% NaCl solution in 3 equivalent doses separated by approximately 3 hr. The dosage referred to below is the total dosage for 3 injections. Control injections were either 0.9% NaCl solution or mannitol diluted in 0.9% NaCl solution. The IUdR preparation used here contains mannitol (50% by weight) to aid in solubility. In our previous publication (5), the weights of IUdR reported were actually the sum of mannitol plus IUdR. In this paper the weights refer to amount of IUdR. The total amount of material used is twice that.

Mitogens

Solutions of Con A (Con A; Calbiochem) and LPS (LPS *E. coli* 055:B5, Westphal preparation; Difco Laboratories, Detroit, Mich.) were prepared in MEM immediately before use and sterilized by filtration.

Cell Preparation

Spleens were removed on the day of test and teased with forceps in MEM, pH 7.0. The suspensions were decanted in 12-ml tubes and allowed to settle for 3 to 5 min. Debris-free supernatants were then removed and the cells were washed, counted, and suspended in desired concentrations. In experiments involving detection of adherent-phagocytic cells, these procedures were performed at 0° in an ice bath.

Detection of B- and T-cells

Ig-Bearing Cells (B-Cells). These were detected with anti-$F(ab)_2$ kindly given by Dr. P. Knopf (Brown University, Providence, R. I.). The $F(ab)_2$ was prepared from myeloma protein of Adj PC-5, an IgG2a, γ 2a, κ tumor. Tumor-bearing mice were used as donors of sera. The fraction, which precipitated at 55% saturation with ammonium sulfate, was dialyzed and subjected to chromatography on carboxymethylcellulose (6). Papain digestion and purification of the fragments were performed as described (6). The antiserum was heat inactivated at 56° for 45 min and absorbed with P3.6.2.8.4./Fab cells, a non-Ig-secreting clone selected from the Adj PC-5 myeloma, and an additional 3 times with C3HeB/FeJ RBC and thymocytes. The antiserum contained 0.02% sodium azide.

θ-bearing Cells (T-cells). These were detected with anti-

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[4] The abbreviations used are: IUdR, 5-iodo-2'-deoxyuridine; Con A, concanavalin A; LPS, *Escherichia coli* lipopolysaccharide; MEM, minimal essential medium; anti-$F(ab)_2$, rabbit antiserum to mouse $F(ab)_2$ immunoglobulin fragments; anti-BAθ, rabbit antiserum to mouse brain-associated θ antigen; SI, stimulation index.

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BAθ. The serum was prepared according to the method of Golub (3) in rabbits preselected for low spontaneous toxicity of serum against C3HeB/FeJ lymphocytes. The serum was heat inactivated at 56° for 45 min and absorbed 4 times with C3HeB/FeJ RBC and bone marrow cells.

Cytotoxicity Assay. This was routinely performed in plastic disposable trays (Limbro Chemical Co. Inc., New Haven, Conn.). Duplicate samples were prepared for each spleen. Five × 10^5 spleen cells in 0.05 ml MEM with 5% heat-inactivated fetal calf serum were incubated at 37° for 45 min with 0.05 ml of test or control serum in the presence of 0.05 ml of guinea pig serum absorbed with agar (2) as source of complement. Serum controls without complement were always made. After incubation the trays were placed on ice, and immediately before counting 0.08 ml of 0.1% trypan blue in MEM was added to each well. The number of live (unstained) and dead (stained) cells were counted and the percentage of dead cells in test wells (*A*) was corrected with percentage of dead cells in serum controls (*B*) according to the formula (7):

$$\frac{A - B}{100 - B} \times 100$$

The number of antiserum-sensitive cells was obtained by multiplying their percentage by the total spleen cell number divided by 100.

A 1:10 dilution of anti-F(ab)$_2$ serum in MEM was found able to kill 45 to 50% spleen cells, 10 to 20% lymph node cells, and 3% thymocytes. Absorption of antiserum with bone marrow cells completely removed the cytotoxicity.

A 1:4 dilution of anti-BAθ in MEM killed 25 to 30% of spleen cells, 65 to 70% lymph node cells, and 95 to 100% thymocytes. Only 3% bone marrow cells were killed, and absorption of the antiserum with thymocytes completely removed the cytotoxicity.

Adherent-Phagocytic Cells

These cells were detected by their ability to adhere to surfaces and incorporate China Ink carbon particles, according to the method of Mantovani *et al.*[5] Five drops of China Ink (4465 Black; Faber-Castell, Newark, N. J.) were added to 1.5 × 10^7 spleen cells in MEM with 10% FCS. One drop was then placed on a hemocytometer, covered with a coverslide, and incubated at 37° for 75 min in humidified air containing 5% CO_2. After incubation, the hemocytometer was immersed in warm 0.9% NaCl solution, and the floating coverslip was removed. The 0.9% NaCl solution was vigorously pipetted over the hemocytometer and adherent-phagocytic (black) cells were counted. With this procedure 5 to 7% adherent-phagocytic cells were found in normal spleens. Conversely, only 0.1 to 0.5% adherent-phagocytic cells were found in suspensions preincubated in Petri dishes at 37° for 75 min.

[5] A. Mantovani, A. Tagliabue, A. Vecchi, and F. Spreafico. Effect of *Corynebacterium parvum* on Cellular and Humoral Antitumoral Immune Effector Mechanisms, submitted for publication.

Lymphocyte Cultures

Duplicate cultures of 10^6 spleen cells in 1 ml MEM containing 5% FCS and antibiotics (streptomycin, 100 μg/ml; penicillin, 100 IU/ml) were prepared for each spleen. Four μg of Con A or 50 μg of LPS in 0.1 ml MEM were added to the cultures, which were incubated in 37° in CO_2 in air atmosphere. Control cultures were made with 0.1 ml MEM. After 48 hr, 0.5 μCi [*methyl*-^{3}H]thymidine (5 Ci/mmole; Amersham/Searle Corp., Arlington Heights, Ill.) were added. Cells were harvested at least 16 hr later by collecting them on Millipore filters (0.8-μm pore size) in a multiple sample collector (Millipore Corp., Bedford, Mass.). The cells were washed with 0.9% NaCl solution and 8 ml of ice-cold 5% trichloroacetic acid. The filters were left to dry overnight in scintillation vials. Five ml of scintillation fluid (Liquifluor; New England Nuclear, Boston, Mass.) were added, and the samples were counted in a Packard Tri-Carb Model 3375 liquid scintillation spectrometer. Data are expressed either as cpm of test cultures minus cpm of control cultures or as SI:

$$\frac{[^3\text{H}]\text{Thymidine incorporation in mitogen-tested cell cultures}}{[^3\text{H}]\text{Thymidine incorporation in control cell cultures}}$$

Clearance of Colloidal Carbon

Colloidal carbon (Pelikan Ink, Hanover, Germany), with an average particle size of 250 Å, was diluted to 8 mg/ml in 0.9% NaCl solution. Mice were incubated in a humid environment at 33–36° for 15 to 30 min and then given i.v. injections of 0.01 ml of the carbon solution per g body weight. At 3-min intervals, 25 μl of blood were drawn from the retroorbital plexus with a calibrated capillary tube. The same tube was used for all bleedings of an individual mouse. It was rinsed in a heparin solution between drawings. The blood sample was added to 2.5 ml distilled H_2O to lyse the cells, and the sample was mixed by vortexing.

The relative amount of carbon present in a sample was determined by spretrophotometry with a Beckman Model DU2 spectrophotometer. Transmittance and absorbance figures were read at 620 nm, using a red filter. The rate of clearance (expressed as absorbance units/min) was determined by reading the samples over a period of 18 min.

RESULTS

Effect of Increasing Doses of IUdR on Lymphoid Cell Levels in the Spleens of C3HeB/FeJ Mice. Normal C3HeB/FeJ mice were given i.p. injections of increasing doses of IUdR on Day 0. Control groups of mice received equivalent doses of mannitol. A 3rd group of mice was given 0.9% NaCl solution. Total spleen cell number and the numbers of anti-F(ab)$_2$-sensitive, anti-BAθ-sensitive, and adherent-phagocytic cells were determined on Day 4. As shown in Chart 1, IUdR, 100 mg/kg, almost doubled the total spleen cell population. Anti-F(ab)$_2$-sensitive cells also increased, but no significant change was observed in the levels of the other 2 cell populations. Smaller and larger doses of IUdR

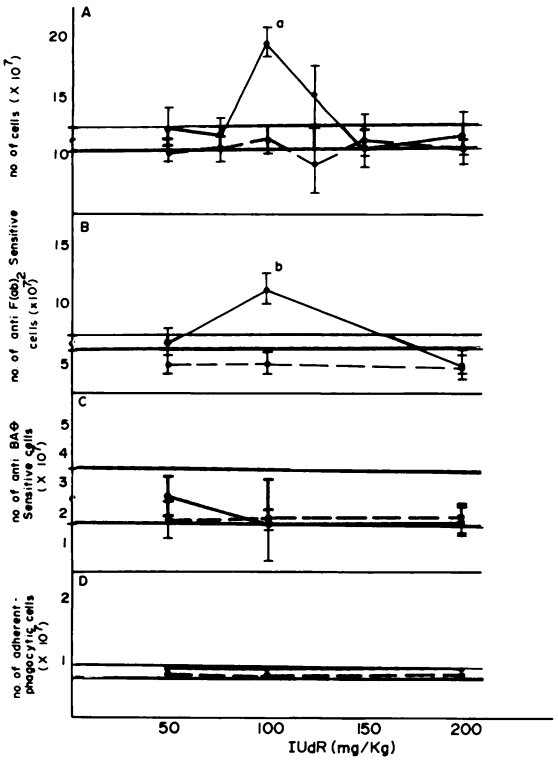

Chart 1. Effect of varying doses of IUdR on spleen cell levels in C3HeB/FeJ male mice (IUdR, Day 0; assay, Day 4). ●—●, IUdR-treated mice; ●- - -●, mannitol-treated mice; ⋯⋯, 0.9% NaCl solution-treated mice; *a*, $p < 0.01$; *b*, $p < 0.001$.

resulted in no changes in number of lymphoid cells in the spleen.

Effect of IUdR on Lymphoid Cell Levels in the Spleen of C3HeB/FeJ Mice on Different Days after Administration. In order to investigate the kinetics of the above effect, IUdR, 100 mg/kg, was injected i.p. into C3HeB/FeJ mice on Day 0, and the numbers of total spleen cells and of lymphoid cell subpopulations were evaluated at subsequent intervals. Spleen cell levels significantly increased on Day 4 only (Chart 2). The number of anti-$F(ab)_2$-sensitive cells increased on the same day, whereas anti-BAθ-sensitive cells and adherent-phagocytic cells did not show any significant change at any time.

In this experiment no 0.9% NaCl solution controls were included, since the previous results with 0.9% NaCl solution were similar to those with mannitol controls (Chart 1).

Effect of IUdR on the *in Vitro* Sensitivity of Spleen Cells to Con A and LPS. Since IUdR increased the number of anti-$F(ab)_2$-sensitive cells, it was of interest to see whether spleen cells from treated mice were more responsive to LPS, a specific mitogen of B-cells. Spleen cells were also tested for their reactivity to Con A in order to detect a possible effect on T-cells, although levels of anti-BAθ-sensitive cells were not affected by the drug treatment.

C3HeB/FeJ mice were treated i.p. with IUdR, 100 mg/kg, on Day 0. On succeeding days their spleens were removed, and the spleen cells were tested for reactivity to either mitogen. As shown in Chart 3, spleen cells from IUdR-treated mice showed an increased sensitivity to LPS over cells from controls on Day 4. A significant difference was found only when data were expressed as SI, but not in mean cpm. This apparent discrepancy can be explained by a relatively lower [^{3}H]thymidine incorporation in nonstimulated spleen cell cultures from IUdR-treated mice as compared to controls. The absolute amount of [^{3}H]thymidine incorporated was not different in LPS-stimulated cultures of the IUdR *versus* the control group.

Surprisingly, on Days 5 to 6, when sensitivity to LPS decreased to normal levels, an augmented response to the mitogenic effect of Con A was observed (Chart 4) as shown by the significant increase in both SI and mean cpm in cultures from IUdR-treated mice, as compared to controls. On Day 7, a normal sensitivity to Con A was again observed.

Effect of IUdR on Phagocytic Function in C3HeB/FeJ

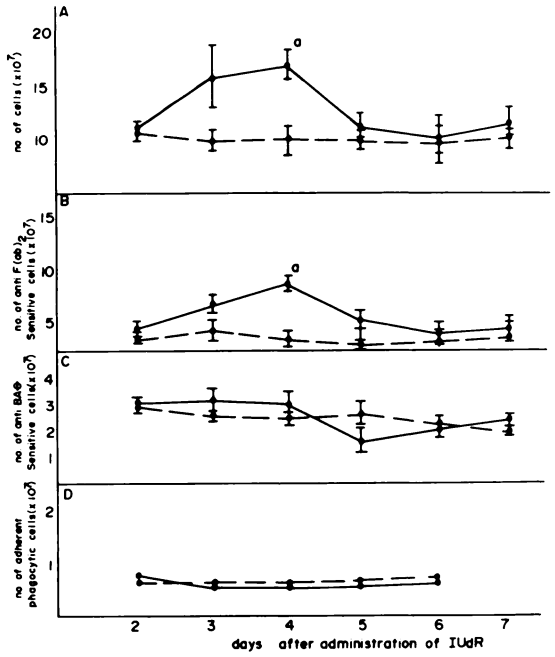

Chart 2. Spleen cell levels in C3HeB/FeJ male mice at various times after administration of IUdR (IUdR, Day 0; 100 mg/kg). ●—●, IUdR-treated mice; ●- - -●, mannitol-treated mice; *a*, $p < 0.01$.

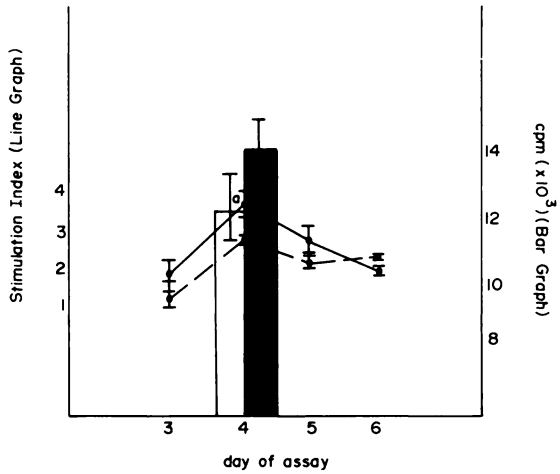

Chart 3. Effect of IUdR on *in vitro* sensitivity of spleen cells to LPS in C3HeB/FeJ male mice (IUdR, Day 0; 100 mg/kg). ■, ●—●, IUdR-treated mice; □, ●- - -●, mannitol-treated mice; *a*, $p < 0.025$.

Mice. As an additional test of the ability of IUdR to affect general immune competence, we investigated the phagocytic function of the reticuloendothelial system. C3HeB/FeJ mice received IUdR, 100 mg/kg, on Day 0. At intervals thereafter they were given i.v. injections of colloidal carbon, and blood samples were taken at 3-min intervals in order to evaluate the relative amounts of residual carbon. A significant increase in the rate of clearing of the carbon particles was seen in IUdR-treated mice on Days 1, 2, and 3. By Day 4 the difference between drug and control groups was gone. A total of 4 experiments were performed. In the 1st 3 experiments the rate of carbon clearing in IUdR-treated mice was compared to that in mice given 0.9% NaCl solution. In the 4th experiment (Table 1), mannitol, 100 mg/kg, was given to control mice. All the experiments gave essentially the same results.

DISCUSSION

The data presented here show that IUdR, 100 mg/kg, increases spleen cell levels 4 days after injection in C3HeB/FeJ mice. This is paralleled by an increase in Ig-bearing spleen cells and also by heightened *in vitro* sensitivity to LPS, a B-cell mitogen. Conversely, no change is apparent in levels of θ-bearing or adherent-phagocytic cells at any time up to 7 days after treatment. However, increased sensitivity to Con A, a T-cell mitogen, occurred on Days 5 and 6, and enhanced phagocytic function, as measured by the rate of clearance of i.v.-injected colloidal carbon, occurred on Days 1 through 3.

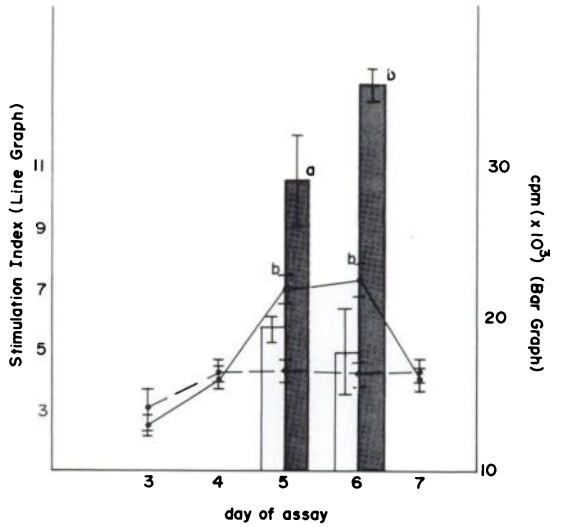

Chart 4. Effect of IUdR on *in vitro* sensitivity of spleen cells to Con A in C3HeB/FeJ male mice (IUdR, Day 0; 100 mg/kg). ■, ●—●, IUdR-treated mice; □, ●- - -●, mannitol-treated mice; *a*, $p < 0.05$; *b*, $p < 0.01$.

Table 1

Effect of IUdR on the rate of clearance of i.v.-injected colloidal carbon in C3HeB/FeJ male mice (IUdR, Day 0; 100 mg/kg)

Treatment	Day of assay	No. of mice	Clearance rate (A/min)
IUdR	1	20	0.026 ± 0.007[a,b]
Mannitol		19	0.018 ± 0.007
IUdR	2	21	0.026 ± 0.006[b]
Mannitol		21	0.020 ± 0.008
IUdR	3	20	0.028 ± 0.006[c]
Mannitol		19	0.017 ± 0.005

[a] Mean ± S.D.
[b] $p < 0.005$, Student's *t* test.
[c] $p < 0.0005$.

A discussion of the mechanisms whereby IUdR might augment immune competence has been presented previously (5). Whether the ability of IUdR to stimulate antibody production to sheep RBC is the consequence of any or all of the changes reported in this paper is problematic. In any event, the increase in nonsensitized mice in total spleen and Ig-bearing cells on Day 4 probably cannot be explained by increased proliferation. The long induction time before the increase, the single B-cell doubling on Day 4, and the relatively short half-life of IUdR (1) seem to rule out a mitogenic activity for the drug. Also, in sheep RBC-immunized mice, IUdR was found to increase the number of hemolysin-plaque-forming cells independently of proliferation (5).

IUdR, a thymidine analog, is incorporated into DNA (4). Because of its possible interference with cell differentiation (8), the B-cell effects reported here may be the result of a "piling up" of Ig-bearing cells due to inhibition of terminal differentiation. An effect on differentiation could also be responsible for the heightened reactivity to Con A, perhaps by altering the proportion of the total T-cell population sensitive to this mitogen. No explanations are thus far available for the stimulation of phagocytic activity by IUdR; it is not known whether this or any other effect is directly due to the drug or is a sequel to effects on other cell populations.

Regardless of the mechanisms responsible for the results observed, the ability of IUdR to modify immune cells and immune competence may have important consequences clinically. Dose, timing, and other parameters of administration will determine whether these consequences are beneficial or harmful to the patient, especially the patient with cancer, whose immunological relationship to his disease may be finely balanced.

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