Carrier- and Hapten-Antibody Producing Cells, in Vitro
II. Effects of Purine Antimetabolites

by T. L. Pazdernik and E. M. Uyeki
Department of Pharmacology, University of Kansas Medical Center, Kansas City, Kansas 66103, USA

Abstract
To determine the effects of purine antimetabolites on the various developmental stages of cells involved in in vitro carrier and hapten responses, purine antimetabolites were added on day 0, day 1, day 2 or day 3 of the in vitro immune response. Anti-HRBC and anti-TNP responses were assessed on day 2, day 3 and day 4 of culture. Our results indicated that purine antimetabolites were: (1) most effective on early and intermediate precursor cell types, (2) most effective when added early during the in vitro immune response and (3) more effective in inhibiting immunogen-stimulated immune response than nonspecific enhancement of the in vitro immune response.

Introduction
Previous studies, in vivo, on the immunosuppressant effect of 6-mercaptopurine (6-MP) on antibody-producing cells (APC) of mouse spleens have indicated a marked dependence on timing of immunogen and 6-MP injections [1-5]. 6-MP was most effective as an immunosuppressant when administered 1 or 2 days after the immunogenic stimulus [2, 3], and has been referred to as a 'class II' immunosuppressive agent by Makinodan et al [1]. However, injections of 6-MP 2 days before the immunogenic stimulus enhanced the formation of APC [4].

To determine which stages of APC development are most sensitive to immunosuppressant agents (i.e., 6-mercaptopurine, puromycin), a model system was developed for assessing immunosuppressant effects on early → intermediate → late precursor cell types involved in in vitro carrier- and hapten-immune responses [6]. The various stages of precursor cells are defined according to kinetic parameters. Mice were immunized with carrier (HRBC), and 3 days later spleen cells were cultured either without an in vitro immunogen or with 2, 4, 6-trinitrophenyl-substituted horse red blood cells (TNP-HRBC). Anti-HRBC and anti-TNP responses were assessed on day 2, day 3 and day 4. Antibody-producing cells detected on day 2 arose mainly from late, on day 3 from intermediate and on day 4 from early precursor cell types. The effects of puromycin have been described previously [7] and this communication describes effects of 6-MP on early → intermediate → late precursor cells of the immune response.

Materials and methods
(1) Animals
Male BDF1 (C57BL/6 x DBA/2) mice between 8 and 16 weeks of age were obtained from Jackson Memorial Laboratories, Bar Marbor, Maine.

(2) Antigens
Sheep red blood cells (SRBC) and horse red blood cells (HRBC) were obtained from Colorado Serum Company Laboratories, Denver, Colorado. 2, 4, 6-Trinitrophenyl-substituted-erythrocytes (TNP-RBC) were prepared by modifications of the procedure of Rittenberg and Pratt [8], as reported by Pazdernik and Uyeki [9].

(3) Tissue culture media
Fetal calf serum was obtained from Reheis Chemical Company, Kankakee, Illinois. Concentrates of minimum essential Eagle's medium (MEM), Hank's balanced salt solution, essential amino acids, non-essential amino acids, sodium pyruvate and guinea-pig complement were obtained from Grand Island Biological Company, Grand Island, New York.

(4) Immunization
BDF1 mice were immunized by intravenous injection of 1 x 10^8 HRBC.

(5) In vitro culture conditions
Spleen cells (1.0-1.2 x 10^7) from normal mice or from mice immunized 3 days previously with 1 x 10^8 HRBC...
(primed spleen cells) were cultured by the method of Mischel and Dutton [10].

(6) Detection of antibody producing cells

Antibody-producing cells were detected as 19S plaque-forming cells (PFC) according to a modification of the Jerne technique [11]. HRBC were used as indicator cells to detect cells producing antibody against the carrier (anti-HRBC response), and TNP-SRBC were used to detect cells producing antibody against the hapten (anti-TNP response).

(7) Drugs

6-Mercaptopurine (6-MP) and azathioprine were obtained from Burroughs Wellcome Company, Tuckahoe, New York. Concentrations of $1 \times 10^{-6}$ M 6-MP and $2 \times 10^{-6}$ M azathioprine per culture were used throughout these experiments.

Results

(1) Effects of 6-MP on nonspecific in vitro enhancement

Both anti-HRBC and anti-TNP responses increased when primed spleen cells were cultured without an in vitro immunogen (see control values in Fig. 1 and 2). We have previously referred to this response as a nonspecific in vitro enhancement of the immune response [6].

Anti-HRBC APC obtained from primed spleen cells cultured without an in vitro immunogen were detected early in the in vitro immune response (days 2–3) (Fig. 1); addition of 6-MP on day 0 or day 1 of culture did not inhibit the nonspecific enhancement of the anti-HRBC response observed on day 2. On the other hand, the day 3 response was inhibited by 6-MP (inhibited 51% when 6-MP was added on day 0). The degree of inhibition was less as the contact time between drug and cells decreased (i.e., 6-MP added on day 1, day 2). Because of the weak anti-HRBC response on day 4, significance of drug effects is questionable at this time period.

Anti-TNP APC detected when primed spleen cells were cultured without an in vitro immunogen were significantly higher than day 0 values ($33 \pm 2$) on day 2 ($517 \pm 30$), day 3 ($1480 \pm 112$) and day 4 ($813 \pm 57$). Like the anti-HRBC response, the anti-TNP response, observed on day 2, also was not inhibited by 6-MP. The day 3 anti-HRBC response was inhibited by 41% when 6-MP was added on day 0; less inhibition was observed when 6-MP was added on day 1 or day 2. Addition of 6-MP on day 0 inhibited the day 4 response by 63%; again a staircase effect was observed (inhibition was less as drug was added progressively later during the in vitro immune response).

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**Figure 1**

HRBC-PFC per culture when $10^7$ HRBC-primed BDF1 spleen cells were cultured without an in vitro immunogen. HRBC-PFC were assessed 2, 3 and 4 days after planting. Control groups were cultured without 6-MP, and $1 \times 10^{-6}$ 6-MP was added to the other groups on days indicated in the bar graphs. Each bar graph represents a mean value obtained from duplicate cultures from three mice. Distance between lines represents two standard errors of the mean.

**Figure 2**

TNP-SRBC-PFC per culture when $10^7$ HRBC-primed BDF1 spleen cells were cultured without an in vitro immunogen. TNP-SRBC-PFC were assessed 2, 3 and 4 days after planting. Control groups were cultured without 6-MP, and $1 \times 10^{-6}$ M was added to the other groups on days indicated in the bar graphs. Each bar graph represents a mean value obtained from duplicate cultures from three mice. Distance between the lines represents two standard errors of the mean.