Comparison of the Immunosuppressive Efficacy of 6-Mercaptopurine, Azathioprine, Cyclophosphamide and 036.5122 (Asta) on the Primary and Secondary Immune Response of Mice to Sheep Erythrocytes

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Abstract
Two alkylating (cyclophosphamide and 036.5122 Asta) and two antiproliferative agents (6-mercaptopurine and azathioprine) have been compared for their immunosuppressive potency on the primary and secondary humoral immune response of mice. If equitoxic dosages of the respective drugs are compared, the alkylating agents proved to be of much higher immunosuppressive potency than the antiproliferative agents. In non toxic dosages alkylating agents were able to completely inhibit a primary or secondary immune response, whilst a similar effect with antiproliferative drugs could not be obtained even within toxic dose ranges. Induction of immunological tolerance was possible only by use of the alkylating agents. The significance of these comparative studies is discussed in view of the frequent use of the tested drugs in clinical immunosuppression.

The immunosuppressive potency of alkylating as well as of antiproliferative agents has been tested by many authors in various experimental conditions (for review see [1]). By use of an appropriate experimental design, with both groups of substances a profound suppression of immune responses could be achieved. Thus, these substances are considered to be potent inhibitors of the immune system and are both widely used in various clinical conditions with the aim to suppress unwanted immune reactions (for review see [2, 3]).

However, at least for antiproliferative agents like 6-mercaptopurine or azathioprine there seems to exist a striking difference in the immunosuppressive effect, if these substances are tested in different species [4]. Thus, the comparative evaluation of immunosuppressive potency within one or between both groups of substances across different species is difficult and may be misleading. For comparative evaluation of efficacy, studies within one species are needed which are done by the use of standardized experimental design and by application of comparable drug dosages. In this paper the antiproliferative agents 6-mercaptopurine and azathioprine and the alkylating agents cyclophosphamide and 036.5122 (Asta), the latter being a newly synthesized cyclophosphamide-analogue [5], are compared in their potency to inhibit a primary or a secondary immune response of adult outbred mice to SRBC. Drug dosages to be compared are based on identical percentages of their respective LD50, and mode of immunization, treatment, and evaluation are kept standardized in all experiments with either drug.

Material and methods

(a) Animals
In all experiments locally bred 2-month-old NMRI mice of both sexes were used. Test animals were kept under conventional conditions and received water and food (Altromin) ad lib.

(b) Immunization
SRBC for primary or secondary immunization were obtained from BAG/Lich (Germany) in Alsever solution, stored not longer than 3 weeks. Before application the cells were washed three times in Ringer solution and adjusted to 10^9 or 10^10 SRBC/ml according to the chosen experimental design. All injections were done i.p. by a total volume of 0.5 ml.

(c) Drugs
Cyclophosphamide (Cy) and 036.5122 (Asta) (5122), kindly supplied as pure substances by Asta-Werke, Brackwede (Germany), were dissolved in Ringer solution. The various concentrations calculated per kg body weight were adjusted in a way, that 0.1 ml had to be injected per 10 g body weight. 6-Mercaptopurine (6-MP) and azathioprine (Aza) were kindly provided as pure substances by Burroughs Wellcome GmbH, Großburgwedel (Germany). In

1) Supported by Deutsche Forschungsgemeinschaft (SFB 107, Mainz).
slight modification of the procedure introduced by BERENBAUM [6] both compounds were suspended for injection in 0.6% hydroxyethyl cellulose ("Oculose", Dr G. Mann, Chemisch-pharm. Fabrik, Berlin, Germany) in Ringer solution at concentrations that provided the desired dose in 0.1 ml per 10 g body weight. Drug preparations were injected i.p. immediately after solution or suspension.

(d) Toxicity assay

Various doses of the 4 drugs used in these experiments were given i.p. as single injections to different groups of 15-20 2-months-old NMRI mice. The number of survivors were counted daily and the percentage of 30-day survival was used to determine the LD₅₀ value for each drug.

(e) Plaque-test

The enumeration of antibody forming cells (PFC) in the spleens was performed according to the CUNNINGHAM modification [7] of the Jerne plaque technique. 7S PFC were developed and calculated according to the method given by DRESSER and WORTIS [8]. Spleens were removed on day 4 after antigen and the total number of PFC (195 ± 75) was determined. Groups of 6 to 10 mice were used for each dose in each experiment and the appropriate control groups. Plaque values given are based on the arithmetic means of each group and expressed as percentage of immunized but not immunosuppressed control groups.

(f) Antibody titration

Blood was obtained from the test animals at different times after immunization from the retroorbital plexus. Antibody determination was performed by use of the microhemagglutinin method of TAKATSY [9] using the U-form microtiter plates of Greiner Co., Nürtingen (Germany) (for details see [10]).

Results

(a) Toxicity

For the NMRI mice used in these experiments the LD₅₀ as calculated by the 30-day survival rate after single injection of either test drug was the following: 6-mercaptopurine ca. 200 mg/kg; azathioprine ca. 400 mg/kg; cyclophosphamide ca. 400 mg/kg; 036.5122 (Asta) ca. 330 mg/kg. Application of 50% of the LD₅₀ of either drug led to a certain mortality up to the end of the 30-day observation period. Injection of 25% of the LD₅₀ or less of each drug did in no instance result in mortality or clinical symptoms of toxicity.

(b) Timing of immunosuppression

In pilot studies published elsewhere [10, 11] and in accordance to observations of other authors [6, 12, 13], optimal immunosuppression by 6-MP or Aza was obtained, if the drugs were given 48 hours after antigen. An optimal effect of Cy or 5122 was observed if these drugs were given 24 hours after antigen.

(c) Inhibition of the primary immune response

Groups of test animals were immunized by an i.p. injection of 5 × 10⁸ SRBC on day 0. Cy or 5122 were injected 24 hours later, 6-MP and Aza 48 hours after antigen. The drug dosages chosen were comparable on the base of the percentage of the LD₅₀ of either drug.

Table 1 gives the total plaque values obtained on day 4 after immunization, calculated as percentage of total PFC of primary controls. Within the dose ranges applied for each drug a dose-dependent inhibition of the primary immune response can be demonstrated. A suppression to below 1% of the control groups can be obtained by a single injection of 5122 which equals 5% of its LD₅₀. The same effect is seen after an injection of Cy in a dosage equal to 10% of its LD₅₀. In contrast, even the LD₅₀ doses of 6-MP or Aza are not sufficient to suppress the PFC response to below 5% of their controls.

Table 2 gives the total plaque values obtained on day 7 after immunization, again calculated as percentage of appropriate controls. Within the dose ranges tested, there is again a dose-dependent inhibition of the late phase of the primary immune response for each test drug used. A suppression to below 1% of

<table>
<thead>
<tr>
<th>% LD₅₀  1)</th>
<th>6-MP</th>
<th>Aza</th>
<th>Cyclo</th>
<th>5122</th>
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<td>0</td>
</tr>
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</tr>
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<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td>12</td>
</tr>
</tbody>
</table>

1) The drug dosages applied are indicated as percentages of the respective LD₅₀ values. 6-MP and Aza were given as a single dose 48 hours after AG, Cy and 5122 Asta 24 hours after AG.