Effect of 5-Aza-2′-deoxycytidine on Antibody Formation and DNA Synthesis in Rat Spleen

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5-Aza-2′-deoxycytidine which is preferentially taken up by the lymphatic tissue and is incorporated into DNA strongly affects the ability of the immune system to synthesize IgG antibodies against sheep red blood cells without affecting IgM antibody formation. A single dose of the drug results in a prolonged inhibition persisting 14 days after the secondary immunization. The inhibitory effect is dose-dependent and is maximal when 5-aza-2′-deoxycytidine is given 2 days after sheep erythrocytes. The drug affects the utilization of thymidine for the synthesis of DNA in the spleen; under certain conditions the enhancement of the rate of DNA synthesis in the spleen has been observed.

5-Aza-2′-deoxycytidine has a considerable cytostatic activity suppressing the cell growth in AKR mice with lymphatic leukemia, in P 388 leukemia bearing mice, and in L 1210 leukemia [1–3]. The drug is generally incorporated into rapidly proliferating cells and its uptake into lymphatic system is preferential [4, 5].

Recently a modulatory effect of 5-azacytidine on antibody production in rats immunized with sheep red blood cells (SRBC) was observed [6]. Although the mode of action of 5-azacytidine differs from that of its 2′-deoxy derivative [5], we undertook in this study to follow the effect of 5-aza-2′-deoxycytidine on the immune response of rats immunized before or after the administration of the drug. Simultaneously the changes in the rate of DNA synthesis in the spleen were measured.

Materials and Methods

Reagents

[2-^{14}C]Thymidine (24 μCi/μmol) was obtained from the Institute for Research, Production and Uses of Radioisotopes (Prague). 5-Aza-2′-deoxycytidine was prepared by Dr. A. Píškala from the Institute of Organic Chemistry and Biochemistry (Prague). The solution of the drug used for the injection was always prepared fresh to avoid the degradation of the drug. [^{3}H]5-Aza-2′-deoxycytidine (19.5 Ci/mmol) was delivered by Dr. B. Černý from the Isotope Laboratory (Prague-Krč).

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Animals, immunization and antibody assay

In all experiments Wistar albino female rats kept under standard laboratory conditions were used. Both the drug and a 30% suspension of 3 times washed SRBC were injected IP. The time interval between primary and secondary immunization was 28 days. The blood samples were collected by cardial puncture. The haemagglutinin titres were measured using Takatsy microtitrator and are expressed as $-\log_2$ of the highest dilution in which the haemagglutination was still observed. The level of IgG antibodies was determined after the treatment of sera with an equal volume of 0.2 M 2-mercaptoethanol. The data were subjected to analysis of variance and the significance of differences was determined using Duncan test.

Incorporation of thymidine into DNA

[2-^{14}C]Thymidine (2–4 μCi per 0.05–0.1 μmol per animal) was injected IP to groups of 4–6 female rats 2 h before killing. The excised tissues were cooled and immediately homogenized in 2 volumes of water. 3 ml of the homogenates were mixed with 3 ml of 0.4 M $HClO_4$, centrifuged, and repeatedly washed with 0.2 M $HClO_4$. Further procedure and hydrolysis of DNA were carried out as described earlier [7]. Spectroscopically pure [2-^{14}C]-thymine was estimated with a spectrophotometer

Unicam SP 700; the radioactivity was assayed in a Packard liquid scintillation spectrometer. The rate of [2-^{14}C]thymidine incorporation was expressed as the specific radioactivity of [2-^{14}C]thymine isolated from the total tissues DNA (dpm/μmol).

Tissue distribution and incorporation of [^{3}H] 5-aza-2′-deoxycytidine

Tritiated drug (400 μCi/0.02 μmol per animal) was injected IP to groups of 4 female rats (180 g). The animals were killed at different time intervals thereafter by cervical dislocation and bled. The tissues under investigation were removed and homogenized in a cooled glass homogenizer with a tight-fitting Teflon pestle in 10 volumes of cold 0.25 mM KCl. One-ml portions of the homogenates were mixed with 1 ml of 0.4 M $HClO_4$, the suspensions centrifuged (5000 × *g*, 5 min, 2 °C) and sediments 3 times extracted with cold 0.2 M $HClO_4$ to remove the acid soluble low-molecular-weight components. Resulting sediments were extracted at 37 °C with 5 ml of alcohol-ether (3 : 1), dried and dissolved at 80 °C in 1 ml of conc. formic acid. The radioactivity of aliquots and absorbance at 260 nm were measured using liquid scintillation system Isocap/300 (Nuclear Chicago Division) and spectrophotometer Unicam SP 700, respectively.

Results

5-Aza-2′-deoxycytidine administered IP to rats is readily distributed among various tissues. While the highest level of the radioactivity in the acid-soluble low-molecular-weight pool was found (Table I) in the liver, much lower amounts were present in the kidney, the spleen and in the thymus. However, measurements of the radioactivity in the acid-insoluble fraction of the cells revealed different picture. The highest amounts of the drug were incorporated into high-molecular-weight fraction in the spleen and in the thymus (Fig. 1), while the lowest radioactivity was found in the liver nucleic acids.

Table I. Tissue distribution of [^{3}H]5-aza-2′-deoxycytidine in rats [a].

Length of treatment [h]	Tissue, dpm per tissue × 10^{-3}			
	Liver	Kidney	Spleen	Thymus
2.5	22825 ± 115	311 ± 22	288 ± 13	148 ± 4
16	1590 ± 162	250 ± 15	159 ± 9	86 ± 3
24	1368 ± 13	225 ± 24	132 ± 15	83 ± 9
48	1050 ± 28	175 ± 9	103 ± 8	46 ± 6

[a] Groups of 4 female rats (175 – 180 g) received IP [^{3}H]5-aza-2′-deoxycytidine (400 μCi/0.02 μmol per animal) and the radioactivity in the low-molecular-weight pool of various tissues was measured as described in Methods.

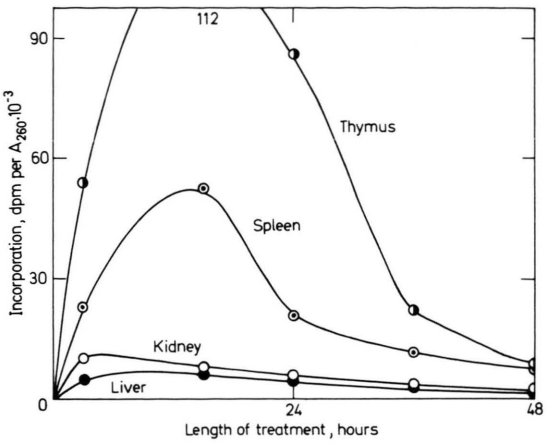

Fig. 1. Time course of [^{3}H]5-aza-2′-deoxycytidine incorporation into DNA in various tissues of rats. Groups of four female rats (180 g) received tritiated drug (400 μCi/0.02 μmol per animal) and its uptake into acid-insoluble fraction of the tissues was measured and is expressed as dpm/A_{260}.

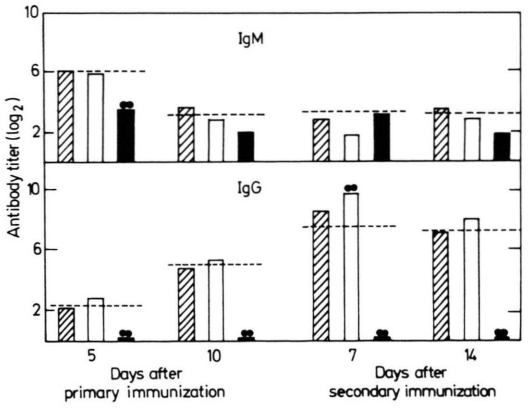

Fig. 2. Changes in IgG and IgM haemagglutinin titres in rats treated with 5-aza-2′-deoxycytidine. Groups of 6–8 female rats (250–300 g) were injected IP with 5-aza-2′-deoxycytidine (20 mg/kg) two days before (▨), simultaneously (□) or two days after (■) primary immunization with 1 ml of 30% sheep red blood cells. Secondary immunization was made 28 days later. IgG and IgM antibody titres were measured on days 5 and 10 after primary immunization and 7 and 14 after secondary one. Mean antibody titres in control animals are given by broken line. Statistically significant differences between experimental and control groups are indicated by black points (one point $P < 0.05$; two points $P < 0.01$).

Table II. Long-term inhibition of IgG haemagglutinins in rats treated with 5-aza-2′-deoxycytidine [a].

Experimental conditions [weeks]	Mean ± SE – $\log_2$ haemagglutinin titres					
	Control animals		Treated animals			
	IgG	IgM	IgG	P-value	IgM	P-value
2° Reaction (4)	6.93 ± 0.26	2.04 ± 0.25	1.43 ± 0.79	<0.01	3.34 ± 0.59	>0.05
2° Reaction (12)	6.98 ± 0.22	1.88 ± 0.19	2.79 ± 0.53	<0.01	2.21 ± 0.30	>0.05
3° Reaction (4 + 12)	6.55 ± 0.39	2.13 ± 0.28	1.56 ± 0.66	<0.01	2.64 ± 0.35	>0.05

[a] Groups of 6 – 8 rats (250 g) were primary immunized with SRBC and 2 days later they were treated with 0.9% NaCl (controls) or 5-aza-2′-deoxycytidine (20 mg per kg). Reimmunizations were carried out 4 or 12 weeks (2° reaction) or 4 and 12 weeks (3° reaction) after the primary immunization. Blood samples for titration were taken 7 days after the last immunization.

The chemical and autoradiographic analyses of the radioactivity incorporated into polymer indicated the incorporation of 5-aza-2′-deoxycytidine into DNA [1].

The preferential uptake of 5-aza-2′-deoxycytidine by the lymphatic tissues and the incorporation of the drug into DNA led us to follow its effect on the synthesis of antibodies stimulated by sheep red blood cells. The results obtained (Fig. 2) indicate a pronounced inhibitory action of 5-aza-2′-deoxycytidine on the formation of IgG antibodies. The maximal effect was observed when the analogue was administered 2 days following immunization. The decline in IgG antibody titres was long-lasting and was observed also after the secondary and tertiary immunization (Table II).

The degree of the inhibitory action of 5-aza-2′-deoxycytidine on the synthesis of IgG antibodies in relation to dose level of the drug is apparent from Fig. 3. The minimal dose of 5-aza-2′-deoxycytidine required to inhibit the synthesis of IgG antibodies significantly (Fig. 3B) was 0.2–0.6 mg/kg. There was a slight but significant enhancement of IgM antibody production when the drug was applied simultaneously with the antigen (Fig. 3A).

Further we followed the changes in the synthesis of DNA in the spleen and the thymus of 5-aza-2′-deoxycytidine treated rats immunized at different time intervals with SRBC. The data summarized in Table III indicate that the rate of DNA synthesis in the spleen and the thymus is not significantly altered during immunization. However, the administration of 5-aza-2′-deoxycytidine simultaneously or shortly after sheep erythrocytes resulted in a marked and reproducible increase of the rate of DNA synthesis 28 days after the drug treatment. The administration

Table III. Synthesis of DNA in the spleen and thymus of immunized rats following 5-aza-2′-deoxycytidine treatment [a].

Experiment (No.)	Treatment during primary immunization	DNA synthesis (dpm/μmol of thymine ± SE)			
		Spleen	[%]	Thymus	[%]
1	Control	2 400 ± 120	(100)	437 ± 73	(100)
	SRBC	2 716 ± 258	(113)	481 ± 37	(110)
	SRBC + 0 day AzadCyd	6 670 ± 312	(278)	782 ± 62	(179)
	SRBC + 2nd day AzadCyd	3 046 ± 480	(127)	507 ± 28	(116)
	SRBC + 4th day AzadCyd	2 416 ± 205	(100)	417 ± 35	(96)
2	Control	3 810 ± 160	(100)	1 578 ± 86	(100)
	SRBC	4 125 ± 355	(108)	1 780 ± 197	(113)
	SRBC + 0 day AzadCyd	13 060 ± 2 320	(343)	3 125 ± 253	(198)
	SRBC + 2nd day AzadCyd	8 410 ± 610	(221)	2 145 ± 110	(136)

[a] Groups of 4 female rats (300 g) immunized with SRBC on days 0 and 21 were injected IP on day 28 with [2-^{14}C]thymidine (Exp. No. 1: 2 μCi/0.05 μmol per animal; Exp. No. 2: 4 μCi/0.1 μmol per animal). 2 h later the animals were killed and DNA synthesis was measured as described in Methods. The rate of DNA synthesis is expressed as the specific radioactivity of thymine isolated from the total DNA in dpm/μmol. 5-Aza-2′-deoxycytidine (AzadCyd) was injected IP at the dose level of 20 mg/kg.

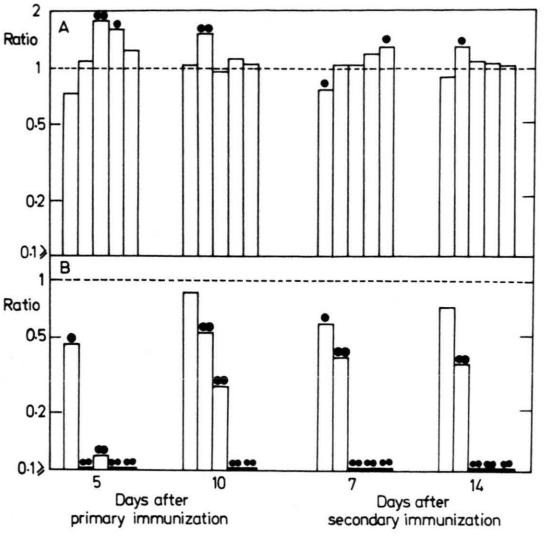

Fig. 3. Effect of increasing doses of 5-aza-2′-deoxycytidine on IgG haemagglutinin titres in rats. Groups of 8 female rats (250–300 g) were immunized with SRBC on days 0 and 28. 5-Aza-2′-deoxycytidine was injected IP either simultaneously (A) or 2 days after (B) primary immunization. Anti-SRBC IgG haemagglutinins were examined on days 5 and 10 after primary immunization and on days 7 and 14 after the secondary challenge. Ratio of mean $-\log_2$ titer in experimental group to mean $-\log_2$ titer in control group is indicated on the vertical axis. Columns 1 to 5 correspond to 0.2, 0.6, 2, 6 and 20 mg/kg of the drug, respectively. Statistical significance is expressed as in Fig. 1.

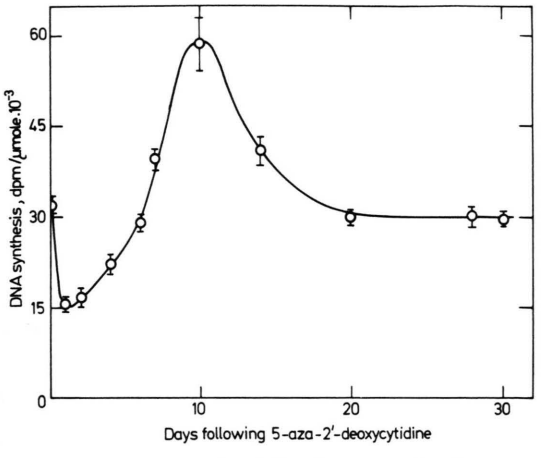

Fig. 4. Variations in thymidine incorporation into DNA in the spleen of 5-aza-2′-deoxycytidine-treated rats. Groups of 4–6 female rats (180 g) received IP 5-aza-2′-deoxycytidine (20 mg per kg) and 2 h before killing [2-^{14}C]-thymidine (2 μCi/0.2 μmol per animal). The rate of DNA synthesis is expressed as dpm/μmol of thymine isolated from the total DNA of the spleen.

of the aza analogue 2 days after immunization, at a moment when the drug-mediated inhibition of IgG antibody synthesis was maximal, had a much lower stimulatory effect on DNA synthesis.

5-Aza-2′-deoxycytidine administered alone caused both the depression and enhancement of thymidine incorporation into DNA in the spleen (Fig. 4). Shortly after the IP injection of the drug the rate of DNA synthesis is depressed more than 50 per cent and thereafter, beginning with the third day, gradually increases. The highest stimulation of thymidine incorporation into spleen DNA was observed in animals pretreated with 5-aza-2′-deoxycytidine 8–14 days before immunization.

Discussion

In the majority of the studies on the immunosuppressive effects of different cytostatics the authors have used repeated administrations at high doses while following the survival of skin grafts or early primary antibody response [8–11]. In our experiments a single dose of 5-aza-2′-deoxycytidine led immediately to an abrogation of IgG antibody production and to a decrease of IgM titres when given 2 days after immunization. Nevertheless, while the IgM antibody titres were quickly restored to normal levels, the production of IgG antibodies remained low for the 12-week period of observation notwithstanding the secondary and tertiary immunization without the additional drug administration.

The remarkable immunosuppressive action of 5-aza-2′-deoxycytidine seems to be related to its selective affinity to the lymphatic system [5], and could be at least partially mediated by its cytotoxic effect against antibody-forming cells, as has been shown for cyclophosphamide [12] or azathioprine [13]. However, the direct cytotoxic effect could have hardly caused the profound and long-lasting decrease of IgG antibody production as observed in our experiments, especially in view to the short half-time of 5-aza-2′-deoxycytidine in blood circulation (4 h). It is likely that the drug affects preferentially specific populations of regulatory *T* lymphocytes. This presumption is supported by the finding that another nucleoside analogue, azathioprine, exerts selective affinity to long-lived *T* cells [14].

5-Azacytidine has also a considerable influence upon the immune response of rats to SRBC [6]. However, there is a remarkable difference between the two drugs: While 5-azacytidine considerably in-

creased IgG antibody production when given simultaneously with antigen, much lower enhancing activity could be demonstrated with the deoxy derivative. Using various doses of 5-aza-2′-deoxycytidine a small increase of IgG titres was observed only exceptionally. On the other hand, 5-aza-2′-deoxycytidine exerts much stronger immunosuppressive effect in comparison with 5-azacytidine when given 2 days after the antigen.

The underlying mechanism of 5-aza-2′-deoxycytidine action on the synthesis of DNA in lymphatic tissues is not yet known [15]. The analogue is preferentially incorporated into DNA of the spleen and thymus (Fig. 1) resulting initially in a decreased and later on in an increased rate of thymidine incorporation into spleen DNA (Fig. 4). The immunization of animals with SRBC simultaneously or 2 days before the administration of 5-aza-2′-deoxycytidine (Table III) induced a significant enhancement of thymidine uptake into DNA both in the spleen and in the thymus of the treated animals. Immunization alone caused only slight and non-significant increase of thymidine incorporation into DNA measured 4 weeks later.

While 5-aza-2′-deoxycytidine administration leads to a marked depression of thymidine and thymidylate kinase activities in the spleen and thymus of rats and mice [15] paralleled by the lower incorporation of thymidine into DNA, no inhibitory effect of the analogue was found in regenerating rat livers highly sensitive to the action of 5-azacytidine [7]. It is known [16] that the activity of deoxycytidine kinase is high in the lymphatic tissues and low in the liver; for this reason 5-aza-2′-deoxycytidine is more easily metabolized to higher 5′-phosphates by lymphoid cells than by hepatocytes and consequently it has no effect on liver regeneration. The differences in the levels of deoxycytidine kinase in mammalian tissues seem to be responsible for the selective uptake of the drug by the lymphatic system.

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