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MODE OF ACTION OF METHOTREXATE UPON INSULIN-ANTIBODY FORMATION IN GUINEA PIGS

DAVID R. MAKULU*

Department of Pharmacology, Indiana University School of Medicine, Indianapolis, Ind. 46202, U.S.A.

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Abstract—Methotrexate (MTXT) effectively inhibits insulin-antibody production in guinea pigs when administered from days 5 to 10 (phase I) or from days 20 to 25 (phase III) after initial antigen injection; or for 5 days after secondary antigen injection (phase II). In the present studies, folinic acid factor (FLA) was shown to reverse this inhibition completely, but itself had no demonstrable effect upon antibody formation. The nature of this reversal during phases I and III was shown to be dependent upon the dose of the metabolite administered. 5-Fluoro-2-deoxyuridine (5-FDUR) was as effective as MTXT when administered during phases I and II, but was completely ineffective in phase III. The immunosuppressive action of 5-FDUR was reversible with administered thymidine. In the presence of MTXT (phases I and II), individual nucleosides were either ineffective or only partially effective in reversing the effects of the antifolate. Thymidine with guanosine, adenosine or 2′-deoxyadenosine completely reversed this inhibition. In phase III, adenosine or guanosine separately, but not thymidine, were able to counteract the effect of MTXT. The relative roles in antibody formation of purine and thymidylate synthetic pathways are discussed in terms of the possible mode of action of MTXT upon induction of antibody formation.

PREVIOUS studies in this laboratory[1] have shown that methotrexate (2,4-diamino-N^{10}-methylpteroyl glutamic acid) maximally inhibits antibody production to insulin in guinea pigs when administered daily from days 5 to 10 (phase I) or from days 20 to 25 (phase III) after primary antigen injection, or for 5 days immediately after secondary immunization (phase II). In each case, the inhibitory effects of methotrexate (MTXT) could be reversed by simultaneous injections of folinic acid factor. In the present studies, the effects of nucleosides upon MTXT-induced inhibition of antibody formation during these three phases were investigated in an attempt to assess the relative roles of purine and thymidylate synthetic pathways in the induction of immune responses.

MATERIALS AND METHODS

Animals. Male albino guinea pigs weighing 300–500 g were used throughout.

Drugs. Methotrexate (MTXT) and solutions of calcium leucovorin [folinic acid factor (FLA)] were obtained from Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N.Y. MTXT was dissolved by dropwise addition of 0·1 N NaOH solution, and subsequently the solution was adjusted to pH 7 with 0·1 N HCl.

* Present address: Postdoctoral Fellow, Department of Pharmacology, Yale University, School of Medicine, 333 Cedar St., New Haven, Conn. 06510, U.S.A.

5-Fluoro-2-deoxyuridine (5-FDUR) was kindly donated by Hoffman-La Roche Inc., Nutley, N.J. All nucleosides were purchased from Sigma Chemical Co., St. Louis, Mo. Nucleosides and 5-FDUR were dissolved in phosphate-buffered 0·9% NaCl solution. Fresh solutions of all drugs were made up daily and immediately administered intraperitoneally in a volume of 0·5 ml.

Immunization. The method of immunizing guinea pigs with insulin has been described in detail elsewhere.[2] Essentially, each animal was inoculated on day 0 with 1 mg of porcine insulin (Eli Lilly & Co.) in a water-in-oil emulsion containing Hemophilus pertussis vaccine (Eli Lilly & Co.) as an adjuvant. A second insulin injection (when necessary) was given 40 days later without the bacterial adjuvant. Plasma samples were collected from the animals' ears at selected intervals and plasma antibody activity determined.

Quantitative assay of antibody activity. The technique reported in detail elsewhere[3] was followed in this study. Essentially, it consisted of a 30-min incubation at room temperature of guinea pig plasma with an excess of unlabeled porcine insulin containing a trace (5 per cent or less) of I^{125}-labeled insulin of the same species (Nuclear Cambridge, Mass.). Antibody–antigen complexes were precipitated with ethanol (*ca.* 76 per cent) and γ-radioactivity in the precipitates determined in a well-type scintillation counter. Antibody activity was calculated as follows: $I_b = I_a (C_s - C_{b1})/V(C_{Exc} - C_{b1})$ where I_b is the insulin bound (μU/μl plasma), I_a is the total amount of insulin added to each tube (μU), V is the volume of incubated guinea pig plasma (μl), and C_{Exc}, C_s and C_{b1} represent observed counts in tubes containing excess antiserum, the sample of guinea pig plasma, and no antiserum respectively.

Statistical analyses of experimental results were carried out according to methods described by Snedecor and Cochran.[4]

RESULTS

Reversal of MTXT inhibition by FLA

Groups of guinea pigs were injected with MTXT (5 mg/kg) between days 5 and 10 (expt. 1) and between days 20 and 25 (expt. 2) with and without graded doses of FLA. The results are summarized in Table 1.

Days 5–10. Animals injected with MTXT (expt. 1; group 2) had significantly lower plasma antibody activity on day 35 than that observed in control animals (group 1). In groups of animals injected with MTXT concurrently with graded amounts of FLA during the same period (groups 3–6), antibody activity in plasma of animals supplemented with 0·05 mg or less of FLA was not significantly different ($P < 0·05$) from that of animals injected with MTXT alone (group 2). Injection of FLA (5·0 mg/kg) with (group 5) or without (group 6) MTXT resulted in production of antibody activity equal to that in animals injected with the antigen alone ($P > 0·5$).

Days 20–25. Plasma antibody activity in animals supplemented with all doses (0·01–10·0 mg/kg) of FLA (expt. 2; groups 9–11) was significantly higher on day 35 than that found in animals that had been treated with MTXT alone (group 8). Antibody activity in the plasma of guinea pigs receiving the highest dose of FLA (10·0 mg/kg) (group 11) was equal to that observed in control animals (group 7).

Effects of MTXT and 5-fluoro-2-deoxyuridine (5-FDUR) upon antibody formation

In a single experiment (expt. 3), groups of animals were injected daily between

TABLE 1. EFFECT OF FLA (N^5-FORMYL FH_4) DOSAGE UPON MTXT-INHIBITED INDUCTION OF ANTIBODY FORMATION*

Expt. no.	Group no.	Drugs administered: Period (days)	MTXT (mg/kg)	FLA (mg/kg)	Antibody activity (day 35)
1	1	5–10	nil	nil	77 ± 7
	2	5–10	5	nil	8 ± 1†
	3	5–10	5	0·005	12 ± 3†
	4	5–10	5	0·050	20 ± 5†
	5	5–10	5	5·00	69 ± 7
	6	5–10	nil	5·00	70 ± 7
2	7	20–25	nil	nil	108 ± 12
	8	20–25	5	nil	20 ± 7†
	9	20–25	5	0·01	52 ± 13†
	10	20–25	5	1·00	104 ± 20
	11	20–25	5	10·00	123 ± 21

* After immunization (day 0), animals (6–7) were injected with MTXT (5 mg/kg of body wt.) concurrently with FLA (0–10 mg/kg of body wt.) daily from days 5 to 10 (expt. no. 1) and from days 20 to 25 (expt. no. 2). Antibody activities (mean ± S. E.; μU insulin bound/μl plasma) were determined on day 35.

† Values that are significantly different from those of control animals ($P < 0·05$).

TABLE 2. EFFECTS OF MTXT AND 5-FDUR (5-FLUORO-2-DEOXYURIDINE) UPON PRIMARY AND SECONDARY ANTIBODY FORMATION*

Group no.	Drugs	Days of daily injections	Antibody activities: Day 20	Δ(20–30)	Δ(30–40)	Δ(40–50)
12	nil		17 ± 3	+91 ± 5	+28 ± 5	+106 ± 8
13	MTXT	5–10	1 ± 0·3	+3 ± 1	+12 ± 4	+90 ± 4
14	5-FDUR	5–10	4 ± 1	+6 ± 2	+20 ± 6	+75 ± 10
15	5-FDUR+ Tdr	5–10	14 ± 3	+101 ± 15	+46 ± 12	+88 ± 11
16	MTXT	20–25	14 ± 3	+12 ± 3	+46 ± 9	+161 ± 16
17	5-FDUR	20–25	16 ± 3	+104 ± 3	+19 ± 6	+103 ± 17
18	MTXT	40–45	12 ± 4	+94 ± 10	+35 ± 14	+12 ± 4
19	5-FDUR	40–45	16 ± 3	+90 ± 8	+35 ± 13	+5 ± 4
20	5-FDUR+ Tdr	40–45	14 ± 6	+113 ± 14	+43 ± 17	+88 ± 19

* Groups of animals (6–7) were given an initial (day 0) and a secondary (day 40) antigen inoculation. Methotrexate (5 mg/kg of body wt.) or 5-FDUR (10 mg/kg of body wt.) were administered to separate groups daily from days 5 to 10 (groups 13–15), from days 20 to 25 (groups 16 and 17) or from days 40 to 45 (groups 18 and 19). Two groups of animals (groups 15 and 20) were injected with additional thymidine (Tdr, 10 mg/kg of body wt.) concurrently with 5-FDUR. Absolute antibody activities (mean ± S. E.; μU insulin bound/μl plasma) at 20 days and increments in activities between days 20 and 30, 30 and 40, and 40 and 50 are shown for each group of animals. Significant differences are discussed in the text.

days 5 and 10 (groups 13–15), days 20 and 25 (groups 16 and 17) or between days 40 and 45 (groups 18–20) with either MTXT or 5-FDUR alone or with 5-FDUR and thymidine. The results are shown in Table 2.

Days 5–10. When MTXT (5 mg/kg) or 5-FDUR (10 mg/kg) was administered daily from days 5 to 10 after initial antigen inoculation (Table 2, groups 13 and 14 respectively) plasma antibody activity on day 20 and the increment in the activity between days 20 and 30 were found to be significantly lower than that observed in control animals (group 12). Animals injected with 5-FDUR concurrently with thymidine (Tdr, 5 mg/kg) over the same period (group 15) responded to the same extent as control animals. After secondary antigen injection on day 40, all these groups of animals produced significant secondary antibody activity (increment between days 40 and 50) equal to that observed in the plasma of control animals ($P > 0{\cdot}5$).

Days 20–25. Animals injected with MTXT daily from days 20 to 25 (group 16) showed normal antibody activity at day 20, normal increments in activity between days 30 and 40, and normal secondary responses (between days 40 and 50), but significantly lower increments in activity between days 20 and 30. By contrast, animals treated with 5-FDUR during the same period produced (group 17) antibody activity to the same extent as control animals at all times ($P > 0{\cdot}5$).

Days 40–45. After an equal primary response to the antigen in all groups (18–20), insignificant increase in secondary antibody activity between days 40 and 50 were observed in animals injected with MTXT (group 18) or 5-FDUR (group 19) for 5 days beginning on the day of the second antigen injection (day 40). By contrast, animals that had been treated with 5-FDUR concurrently with thymidine (5 mg/kg) during the same period (group 20) produced secondary antibody activity equal to that observed in the plasma of control animals ($P > 0{\cdot}4$).

From this study, it was concluded that whereas MTXT inhibits antibody formation administered during all three phases, 5-FDUR is only effective when given during the early part of primary or secondary responses; and that the inhibitory effect of 5-FDUR is reversible with concurrently administered thymidine.

Effects of concurrent administration of MTXT and nucleosides

The effects of nucleosides upon MTXT-suppressed antibody formation depended upon the times of administration, their identities and the combinations in which they were given. Results are summarized in Table 3.

Days 5–10. After an initial antigen injection (day 0), MTXT was administered to animals daily from days 5 to 10 with or without individual or multiple nucleosides (Table 3; expt. 4). Primary antibody activity (increment between days 20 and 25) was significantly lower in the plasma of animals injected with MTXT alone. Animals injected with MTXT concurrently with either 2′-deoxyadenosine (5 mg/kg) or thymidine (5 mg/kg) produced antibody activity which was significantly less than that produced by control animals.

Treatment of animals with MTXT concurrently with FLA (5 mg/kg) or a combination of thymidine with adenosine, 2′-deoxyadenosine or guanosine induced antibody production equal to that obtained in control animals ($P > 0{\cdot}5$). In experiments not reported here, guanosine, adenosine or 2′-deoxycytidine, when injected individually but concurrently with MTXT, yielded plasma anitbody activity which was not significantly different from that observed in animals injected with MTXT alone.

TABLE 3. EFFECTS OF PURINE AND PYRIMIDINE NUCLEOSIDES UPON MTXT-SUPPRESSED ANTIBODY FORMATION*

Expt. no.		4	5	6
			Days	
Period of drug admin.		5–10 (Phase I)	20–25 (Phase III)	40–45 (Phase II)
Immune response period		20–25	20–30	40–50
Drugs administered				
MTXT (5 mg/kg)	Metabolites (5 mg/kg)			
nil	nil	+85 ± 4	+160 ± 6	+124 ± 6
+	nil	+14 ± 3†	+15 ± 7†	+21 ± 6†
+	Adenosine		+136 ± 6	
+	Guanosine		+126 ± 4	
+	Deoxyadenosine	+49 ± 4†	+31 ± 5†	+42 ± 13†
+	Tdr	+19 ± 5†	+16 ± 2†	+30 ± 10†
+	Tdr + Adenosine	+87 ± 5		
+	Tdr + Deoxyadenosine	+66 ± 8	+ 41 ± 4†	+ 84 ± 28†
+	Tdr + Guanosine	+82 ± 5		+113 ± 17
+	N^5-Formyl FH_4	+72 ± 2		+119 ± 24

* Groups of 7–8 animals (in 3 experiments) were inoculated with an antigen on day 0 and, when necessary, on day 40. Methotrexate was administered with or without metabolites from days 5 to 10 (expt. 4), days 20 to 25 (expt. 5) or (in animals given a secondary antigen injection) from days 40 to 45 (expt. 6). Antibody activities are reported as increments in activities between days 20 and 25, 20 and 30, 40 and 50 (mean ± S. E.; μU insulin bound/μl plasma).
† Values that are significantly different from those of control animals.

Days 20–25. Animals that had been injected with MTXT daily from days 20 to 25 (Table 3; expt. 5) after antigen injection showed significantly lower increments in antibody activity between days 20 and 30 compared with that found in the plasma of their control counterparts. Injection of MTXT simultaneously with thymidine, 2'-deoxyadenosine or both induced insignificant ($P > 0{\cdot}5$) production of antibody above that observed in animals that had been injected with MTXT alone. However, animals that had been injected with MTXT with either adenosine or guanosine produced antibody activity to the same extent as their control counterparts ($P > 0{\cdot}4$).

Days 40–45. After primary (day 0) and secondary (day 40) antigen injection, MTXT was administered to animals for 5 days from day 40, with or without various nucleosides (Table 3; expt. 6). Secondary antibody activity (increment between days 40 and 50) observed in the plasma of animals that had been treated with MTXT concurrently with thymidine or deoxyadenosine was not significantly higher than that found in animals treated with MTXT alone but was significantly lower than that observed in the plasma of control animals ($P < 0{\cdot}001$). When compared with activity in control animals, secondary antibody activity was not significantly different in plasma of animals injected with MTXT concurrently with thymidine and deoxyadenosine ($P > 0{\cdot}2$), with thymidine and deoxyguanosine ($P > 0{\cdot}5$) or with FLA ($P > 0{\cdot}5$).

Reversal of MTXT effects by additional graded doses of thymidine and deoxyadenosine

Animals were injected with MTXT (5 mg/kg of body wt.) and 2'-deoxyadenosine (10 mg/kg, body wt.) with or without graded amounts of thymidine (0–10 mg/kg of

TABLE 4. REVERSAL OF MTXT EFFECTS BY ADDITIONAL GRADED DOSES OF THYMIDINE AND DEOXYADENOSINE*

Expt. no.	Group no.	Drugs administered (mg/kg)			Primary response
		MTXT	2′-Deoxyadenosine	Thymidine	
7	21	nil	nil	nil	110 ± 8
	22	5	nil	nil	36 ± 4†
	23	5	10	nil	44 ± 7†
	24	5	10	0·005	53 ± 11†
	25	5	10	0·5	80 ± 11†
	26	5	10	5·0	99 ± 7
	27	5	10	10·0	95 ± 11
8	28	nil	nil	nil	131 ± 14
	29	5	nil	nil	1 ± 1†
	30	5	nil	10·0	1 ± 1†
	31	5	0·005	10·0	6 ± 2†
	32	5	0·50	10·0	65 ± 11†
	33	5	5·0	10·0	78 ± 12†
	34	5	10·0	10·0	118 ± 14

* After antigen inoculation (day 0), MTXT (5 mg/kg of body wt.) was administered daily from days 5 to 10, with (expt. 7) 2′-deoxyadenosine (10 mg/kg of body wt.) and thymidine (0–10 mg/kg of body wt.) or (expt. 8) 2′-deoxyadenosine (0–10 mg/kg body wt.) and thymidine (10 mg/kg of body wt.). Antibody activities (mean ± S. E.; μU insulin bound/μl plasma; N = 6–7) were determined on day 35.

† Values that are significantly different from those observed in control animals.

body wt.) from days 5 to 10 after initial antigen inoculation (Table 4; expt. 7). In the presence of MTXT alone (group 22), significantly lower antibody activity was found on day 35 compared with levels observed in control animals (group 21). In the presence of MTXT and 2′-deoxyadenosine with (groups 24 and 25) or without (group 23) 0·5 mg or less of thymidine, antibody activity was not significantly different from that produced by animals treated with MTXT alone. Doses of thymidine higher than 0·5 mg induced significantly higher antibody production, 5 and 10 mg of thymidine (groups 26 and 27 respectively) yielding antibody activity comparable to that observed in plasma or control animals ($P > 0·05$).

In a separate experiment (expt. 8), MTXT was administered daily from days 5 to 10 either alone or with thymidine (10 mg/kg of body wt.). Graded doses of 2′-deoxyadenosine (0–10 mg/kg) were given to various groups of animals. Insignificant antibody activity was observed on day 35, in animals treated with MTXT alone (group 29), MTXT and thymidine with (group 31) or without (group 30) 0·005 mg/kg of 2′-deoxyadenosine. In the presence of thymidine and additional 0·5 mg/kg of the purine nucleoside (group 32), significant antibody production was observed, although this activity was significantly lower than that found in the plasma of control animals. Animals injected with 5 or with 10 mg/kg of 2′-deoxyadenosine (groups 33 and 34 respectively) produced correspondingly higher antibody activity, the last dose inducing antibody production comparable with that observed in control animals ($P > 0·1$).

DISCUSSION

The role and importance of cellular proliferation in the induction of an immune response has long been recognized and has been the subject of a recent review.[5] It is now generally accepted that an antigen, or a modification of it, initially stimulates proliferation of pre-existing antigen-sensitive cells, the progeny of which eventually matures into antibody-forming cells. It is postulated that transformation of stimulated antigen-sensitive cells into mature antibody-forming cells does not occur without proliferation. This scheme will form the basis for the discussion of results obtained in the present studies. It would therefore be expected that drugs such as folic acid antagonists, which interfere with synthesis of DNA, would have profound inhibitory action upon induction of an immune response, and that this effect would be greatly influenced by relative timing of drug and antigen administration. The over-all effect would therefore be related to the stage of differentiation and proliferation of cells in lymphoid organs at the time of drug administration.

In previous studies[1] it was shown that maximum inhibition of insulin-antibody formation in guinea pigs was achieved if MTXT was administered from days 5 to 10 (phase I) after the day of immunization. Given between days 20 and 25 (phase III), when antibodies would normally begin to appear in the circulation, MTXT induced complete but temporary inhibition, normal antibody formation resuming after cessation of drug treatment. MTXT also inhibited secondary antibody formation when given for 5 days beginning on the day of secondary antigen inoculation (phase II).

As demonstrated in previous studies[1] and confirmed in the present work (Table 1), FLA (N^5-formyltetrahydrofolate) can reverse the action of MTXT when the two compounds are administered concurrently; FLA itself has no demonstrable effect upon induction of antibody formation. When administered during phases I and III (Table 1), reversal of the action of MTXT is related to the dose of FLA injected, although no clear-cut dose–response relationship was established under the conditions used in the present studies. A similar observation was reported by Jacobson[6] who found no well-defined relationship between the quantity of FLA and the restoration of aminopterin-inhibited growth of cultured fibroblasts. However, it is clear from present studies that when administered in adequate doses FLA can completely and quantitatively prevent the effects of MTXT in all sensitive phases of the immune response, suggesting that under these conditions MTXT is exerting its biological effects by interference with the formation of active coenzymes from folic acid. This conclusion contrasts with suggestions by other workers[7,8] who postulate that the drug inhibits immune responses in the guinea pig by mechanisms which are independent of any action upon folic acid metabolism.

On the basis of previous experiments cited above, it is postulated that phases I and II of this immune response are periods of stimulated nucleic acid synthesis in antigen-stimulated cells. Further supportive evidence for this contention comes from studies in which 5-fluoro-2-deoxyuridine (5-FDUR), an analogue of thymidine, was shown to inhibit antibody formation when administered during phases I and II (Table 2; groups 14 and 19 respectively). Inhibition with this drug is reversible by simultaneously administered 2′-deoxythymidine during both phases (groups 15 and 20 respectively), suggesting that inhibition of DNA synthesis by interference with the source of Tdr, is the primary mode of immunosuppression by 5-FUDR during phases I and II. By contrast, the pyrimidine analogue has no inhibitory effect upon antibody formation

when administered during the late phase of the primary response (phase III; days 20–25; Table 2, group 17). This observation suggests that Tdr, and therefore DNA synthesis, are not required for antibody formation during this phase of the immune response and that the mode of immunosuppression observed with MTXT must be mediated via a process other than DNA synthesis. It is worth noting that, as shown with MTXT in earlier experiments[1] and confirmed here, animals in which 5-FDUR induced almost complete inhibition of primary antibody formation responded in a characteristic anamnestic fashion when challenged with a secondary antigen inoculation, suggesting that these drugs have no effect upon induction of "immunological memory".

Because MTXT prevents the conversion of folic acid through tetrahydrofolic acid to the active coenzymes, it should be possible to prevent the action of the drug by addition of those metabolites whose synthesis is dependent upon folate coenzymes. When administered individually but concurrently with MTXT (Table 3) during phase I, Tdr (expt. 4), guanosine, adenosine and 2′-deoxyadenosine were each ineffective in reversing the action of MTXT; but 2′-deoxyadenosine partially prevented the inhibitory effect (expt. 4). Complete reversal of the MTXT effects was achieved with Tdr in the presence of either deoxyadenosine, adenosine or guanosine. This suggests that Tdr and a source of purine must be present to counteract the action of MTXT. This effect is also true in the secondary response (phase II; expt. 6). Results indicate that under these conditions MTXT exerts its biological effects through inhibition of thymidylate and purine nucleotide biosynthesis. FLA can reverse this inhibition, as can the administration of Tdr (a source of thymidylate) plus a source of purine nucleotides.

In phase III (expt. 5), deoxyadenosine by itself induced some reversal but adenosine and guanosine were each able to reverse completely the effects of MTXT. On the other hand, Tdr even in the presence of deoxyadenosine was ineffective in preventing the action of MTXT. This observation suggests that the inhibitory action of MTXT during this period results not from inhibition of DNA replication but from inhibition of purine biosynthesis. This hypothesis is compatible with the well-known fact that cells that are actively synthesizing antibodies, unlike their primitive precursors, are mature lymphocytes with very low rates of nucleic acid synthesis. It also explains the lack of inhibitory effect by 5-FUDR and the ineffectiveness of Tdr in reversing the action of MTXT upon antibody formation during this phase. On the other hand, purine nucleotides are essential for actual antibody synthesis since this process proceeds by mechanisms shared in common with other protein-synthesizing systems.[9]

Having established qualitatively the requirement for Tdr and a source of purine nucleotides to prevent the action of MTXT during the early parts of primary and secondary responses (phases I and II), efforts were made to separate these two requirements by studying the effects of graded doses of the one in the presence of an excess of the other. Within the limits of the system *in vivo*, the degree of reversal in the presence of a high dose of deoxyadenosine (Table 4, expt. 7) was directly proportional to the quantity of Tdr administered. Likewise, in the presence of a fixed but high dose of Tdr (Table 4, expt. 8) deoxyadenosine induced reversal of the MTXT effect, which was related to the dose of the metabolite administered.

The dependence of antibody formation upon thymidylate when a source of purine nucleotides is available is explicable on the basis that thymine is required (as a component of DNA) for DNA synthesis and subsequent cellular proliferation of antigen-

stimulated cells. However, the dependence upon a source of purine nucleotides when thymidylate is present in adequate amounts may stem from two points. First, purines are components of nucleic acids and therefore are essential for cellular proliferation. Secondly, it has been established that among important reactions involved in DNA synthesis are those catalyzed by thymidine kinase (ATP: thymidine 5′-phosphotransferase, EC 2.7.1.21), and thymidine monophosphate kinase. (ATP: thymidine monophosphate phosphotransferase, EC 2.7.4.9); and that the activities of these enzymes appear to be regulated by the presence and concentration of adenine nucleotides both *in vitro*[10] and *in vivo*.[11] The over-all effect is that purine nucleotides have profound influence upon the utilization of Tdr in the synthesis of DNA.

It appears significant that injected nucleosides can reverse the immunosuppressive action of MTXT, in view of the fact that these compounds, particularly pyrimidine nucleosides, are normally rapidly degraded and can therefore not contribute materially to metabolic pathways. A plausible explanation for this seemingly paradoxical observation is that the cellular proliferative phase of the immune response is attended with certain biochemical changes in the cells involved. It is known that immune response to an antigen involves many organs such as lymph nodes, spleen, liver, lung, etc.,[9] and that under conditions of stimulated cellular proliferation nucleic acid synthetic pathways in tissues become dominant over degradative pathways in tissues involved. Stimuli for such cellular proliferation include partial hepatectomy,[12] injury,[13] and injections of hormones,[14] phytohemaglutinin[15] or foreign serum proteins.[16] Under these conditions, injected Tdr has been shown to be utilized in macromolecular synthesis instead of being degraded.[17] In studies reported recently by Fischer *et al.*,[18] cytidine (a pyrimidine nucleoside which is normally degraded rapidly when injected into animals) was shown to effectively prevent the strong immunosuppressive effects of its antagonist, cytosine arabinoside, when the two compounds were injected concurrently in mice, 3 or 4 days after antigen injection. Purine nucleotides, on the other hand, have been shown to participate in metabolic pathways when administered to animals. Thus dietary adenine was purported to serve as a precursor for nucleic acid synthesis in normal rodent tissues.[19,20] Likewise, Fieldman *et al.*[21] showed that immunosuppressive effects of 6-mercaptopurine can be reversed with injected DNA and RNA digests. Since no transport mechanism for nucleotides across cell membranes is known to exist, it can be assumed that injected nucleic acid digests are first broken down extracellularly to nucleosides which then enter cells to compete against the analogue.

From these studies, it is concluded that MTXT suppresses immune responses by inhibiting one or both purine and thymidylate biosynthetic pathways, depending upon the stage of the immune response. These studies *in vivo*, in agreement with studies *in vitro* reported by Borsa and Whitmore,[22] show that the involvement of the two pathways can be separated and their relative sensitivities and roles in immune responses or growth of tissues can be assessed through the use of MTXT and nucleosides. The hypotheses presented above are tentative since all experiments were carried out *in vivo* and many more factors may be involved. Confirmation will have to await studies *in vitro* in which enzymatic systems of immune cells can be studied.

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