STUDIES ON CYCLOPHOSPHAMIDE-INDUCED TOLERANCE TO SHEEP ERYTHROCYTES*

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Immunological tolerance remains a fundamental but incompletely understood problem in immunology (1, 2). Of the several varieties of such tolerance, that induced by immunosuppressive drugs (3) affords several advantages in the study of the general problem. Immunosuppressive drugs produce tolerance to a variety of antigens in adult animals under conditions that can be controlled and varied (4). Further, these drugs produce metabolic alterations which, though multiple, are well-known, and can therefore be correlated with the resulting immune suppression.

The present study concerns immunological tolerance to sheep erythrocytes produced in CBA mice with the immunosuppressive drug cyclophosphamide (Cytoxan). This cytotoxic alkylating agent is effective in the mouse, producing complete tolerance at dosage well below that which causes any fatal toxicity (5, 6). Employing sheep erythrocytes and the Jerne plaque technique (7, 8), it is possible to obtain information about the cellular kinetics of developing tolerance and recovery there-from which is unavailable from antibody methods. With isotopic methods, deoxyribonucleic acid and ribonucleic acid synthesis can be measured and correlated with the induction of tolerance. Finally, because both the clearing of foreign erythrocytes from the blood (9, 10) and the cytoxic effect of cyclophosphamide (11) are prompt, the chronology of events can be established with precision. This paper deals with tolerance induction in this system, and a subsequent one will consider recovery from tolerance and the effect of the thymus.

Methods

Animals and Materials.—Female CBA mice 10–14 wk of age obtained from the Jackson Laboratory, Bar Harbor, Maine, were used. Cyclophosphamide (Cytoxan, Mead Johnson Laboratories, Evansville, Ind.) was injected intraperitoneally as a freshly made saline solution containing 5 mg per ml. The usual tolerance-inducing drug dose was 330 mg per kg given either as a single dose or as four daily divided doses. Unless otherwise stated, the tolerance-inducing

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injection of antigen was 0.8 ml of a 30% suspension (6.4×10^9 cells) of once saline-washed fresh sheep cells (obtained weekly from Baltimore Biological Laboratories, Baltimore, Md.) administered intravenously at the same time as the cyclophosphamide. All experimental points in the present study represent the arithmetic mean of at least three animals. Hemagglutination and hemolysin titers were determined with fresh sheep cells in the Microtiter apparatus (12) employing standard methods (13) and an initial serum dilution of 1:10.

Determination of Antibody-Forming Cells.—Hemolysin-producing spleen cells were enumerated by the technique of Jerne et al. (7, 8). 4 days after intravenous challenge with 0.25 ml of 10% sheep cells, animals were sacrificed and the individual spleens sieved with a Cytosieve (Will Scientific, Inc., Cambridge, Mass.) into 2.0 ml of Gey's balanced salt solution, The spleen cell suspension was counted in a hemocytometer and diluted or concentrated to give a convenient number of plaque-forming cells in a 0.1 ml volume (50–1000 plaque-forming cells). One-half ml of 2.5% agarose (SeaKem brand, obtained from Bausch and Lomb Incorporated, Rochester, N. Y., and dissolved in Gey's solution by heating for 1 hr at atmospheric pressure in a steam chamber) solution was added to 1.5 ml of Gey's solution heated to 46°C, and to this were added 0.1 ml of the spleen cell suspension and 0.1 ml of 15% washed sheep cells, both at room temperature. After mixing, this cell suspension was poured into a 100 × 15 mm disposable Petri dish (Falcon) into which a sublayer of 10 ml of 1.2% agarose had been poured the previous day. When the agarose cell suspension had gelled, the dish was incubated at 37°C for 2 hr. The 19S or direct hemolytic plaques were developed by adding complement (1 ml of a 1:10 dilution of lyophilized guinea pig serum obtained from Baltimore Biological Laboratories) and incubating for an additional hour. Indirect or 7S plaques were determined on duplicate plates by the method of Dresser and Wortis (14), employing a rabbit antiserum to mouse gamma globulin (mouse Fraction II, Pentex Incorporated, Kankakee, Ill.). Following the initial 2-hr incubation, plates were incubated for 1 hr with a 1:50 dilution of rabbit antiserum prior to the complement addition. The hemolytic plaques surrounding each antibody-forming cell were enumerated with the aid of a bacteria colony counter (New Brunswick Scientific Co., New Brunswick, N. J.).

Determination of Rates of Deoxyribonucleic Acid (DNA) and Ribonucleic Acid (RNA) Synthesis.—The techniques employed were modified from those described by Dalgarno and Martin (15). Mice to be analyzed for DNA synthesis received 15 μc of thymidine-methyl-^{3}H (28 mc per mg, obtained from New England Nuclear Corp., Boston, Mass.) by intraperitoneal injection 4 hr before sacrifice, while mice to be analyzed for RNA synthesis received 15 μc of uridine-^{3}H (19 mc per mg) 2 hr before sacrifice. The whole spleen was excised, weighed, and homogenized in 8 ml of cold 0.25 M sucrose with a Potter-Elvehjem homogenizer. The nuclear fraction was separated from the cytoplasm by sedimentation at 600 *g* for 10 min in the cold and then resuspended in 8 ml of cold sucrose. 2 ml portions of 1 M perchloric acid were added to the nuclear and to the cytoplasmic fractions. The precipitated nuclear and cytoplasmic fractions were then washed (by centrifugation) three times with cold 0.1 M perchloric acid, extracted with cold 2:1 ether:methanol, defatted for 30 min at room temperature with 1:1 methanol:chloroform, washed twice with ether, and suspended overnight at 37°C in 1 ml of 0.3 M KOH to hydrolyze the RNA. The following morning the KOH solution was chilled and neutralized with 0.63 ml of 20% trichloracetic acid. The precipitated DNA was separated from the RNA in the supernatant by centrifugation, and then washed with 1 ml of cold 5% trichloracetic acid. The combined supernatants were used for RNA determination by the orcinol method (16) and radioactivity measurement by scintillation counting. The DNA precipitate was next suspended in 1 ml of 5% trichloracetic acid and hydrolyzed by heating for 30 min at 90°C in a covered tube. Following centrifugation, the DNA was measured with diphenylamine (17) and counted.

DNA was studied in the nuclear fractions of the animals receiving thymidine, and RNA

was examined in the nuclear and cytoplasmic fractions of animals receiving uridine. ½ ml portions of RNA or DNA digests were counted in 10 ml of dioxane scintillation fluid (200 g scintillation grade naphthalene, 10 g PPO, 0.25 g POPOP, 100 ml toluene per liter of purified dioxane) in a Packard "Tricarb" liquid scintillation spectrometer.

RESULTS

Temporal Relationship of Drug to Antigen for Tolerance Induction.—Fig. 1 presents data from an experiment designed to explore the time relationships of antigen injection and drug administration in the induction of tolerance. Cyclophosphamide was injected intraperitoneally as a single dose of 330 mg per kg or 165 mg per kg on the day of sheep cell administration or on various days before or after the antigen. The tolerance-inducing sheep cell injection was 6.4×10^9 cells (0.8 ml of a 30% suspension), and tolerance was maintained by weekly intraperitoneal injections of 0.2 ml of 10% sheep cells. The data recorded in the figure are the hemagglutination titers 35 days after the initial sheep cell injection.

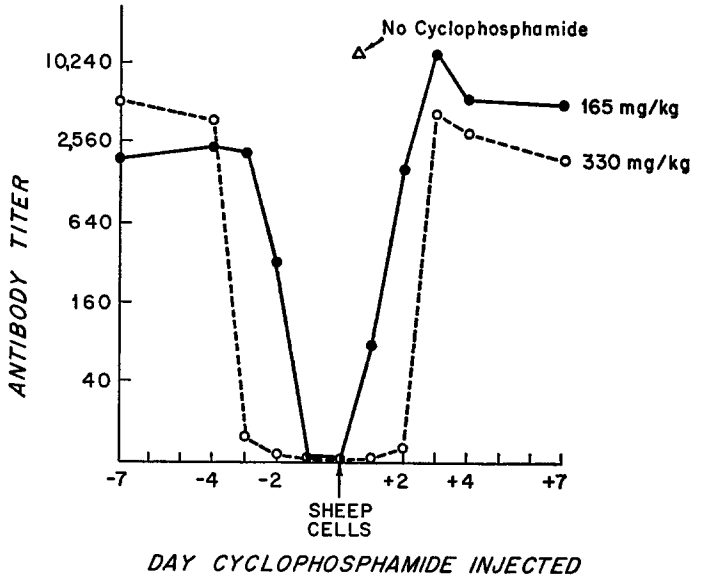

FIG. 1. Relationship of the times of sheep cell injection and cyclophosphamide administration in drug-induced immunological tolerance.

It will be seen from Fig. 1 that a precise relationship exists between the times of antigen and drug injection to produce complete tolerance. Thus at the higher drug dose, complete tolerance is produced when cyclophosphamide is given together with antigen, the day after antigen, and 1 or 2 days before, while

tolerance is almost complete when the drug is given 3 days before antigen or delayed until the 2nd day after. The lower drug dose defines more precisely the period of maximum tolerance induction to the day of antigen and the day immediately prior.

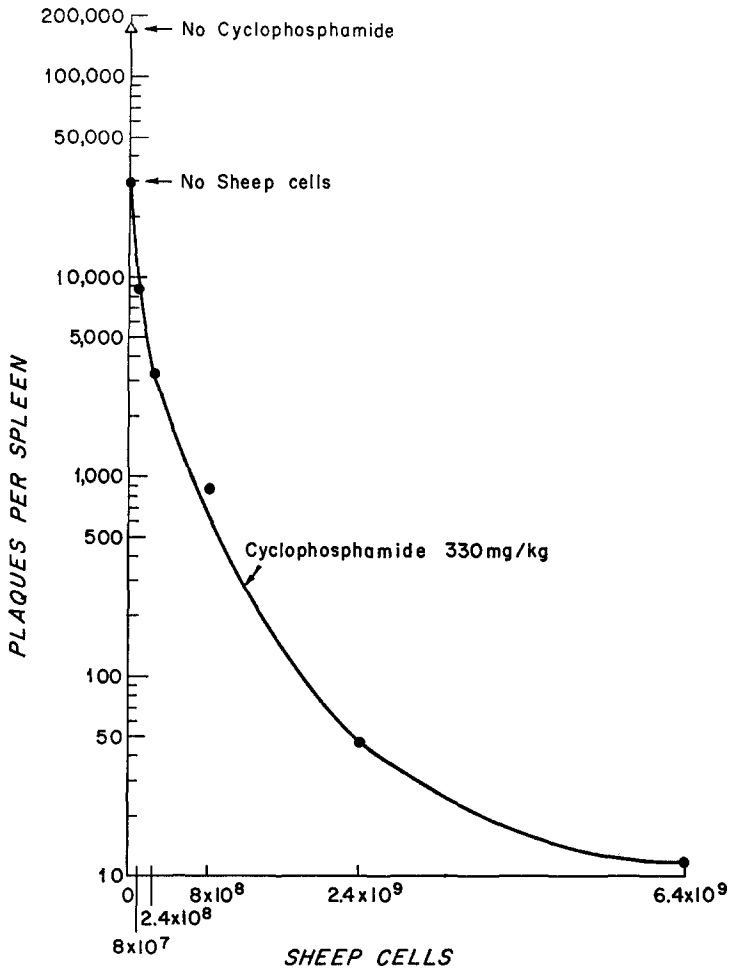

FIG. 2. Influence of antigen dose on cyclophosphamide-induced immunological tolerance. The sheep cell injection was followed immediately by cyclophosphamide.

Antigen Dose.—Fig. 2 presents data on the effect of antigen dose on tolerance induction. In this experiment sheep cells in varying amounts were injected intravenously, followed immediately by a single dose of cyclophosphamide (330 mg per kg). On the 14th day the animals received the usual challenge of sheep cells and the spleens were plaqued 4 days later. It will be noted that the spleens

of animals that received cyclophosphamide *without* sheep cells contain only one-sixth the number of plaque-forming cells of those from animals that received no drug (32,000 plaques compared to 178,000). Furthermore, it is only at the highest sheep cell dose (6.4×10^9 cells) that essentially complete specific immunological tolerance is achieved—reduction of the plaque count to 12 per

TABLE I

DNA and RNA Metabolism in Mouse Spleen at Various Times after Cyclophosphamide Administration

Experiment No.	Day	Cytoxan dose*	DNA			Nuclear RNA			Cytoplasmic RNA		
			μg	cpm	Specific activity	μg	cpm	Specific activity	μg	cpm	Specific activity
1	0	—	1080	9100	8.4	147	996	6.8	197	1070	5.4
	1	330	260	108	0.4	43	210	4.9	101	292	2.9
	2	330	294	175	0.6	37	178	4.8	84	399	4.8
	3	330	254	115	0.5	35	156	4.4	144	430	3.0
	4	330	235	330	1.4	32	180	5.7	140	722	5.1
	5	330	200	340	1.7	52	240	4.6	137	—	—
	6	330	394	3070	7.9	113	440	4.0	182	970	7.1
2	0	—	980	5314	5.4	125	391	3.1	149	474	3.2
	1	165	517	227	0.4	95	213	2.2	137	172	1.3
	1	82	739	2091	2.8	109	343	3.1	149	302	2.0
	2	165	436	556	1.3	65	135	2.0	116	225	1.9
	2	82	647	1202	1.9	78	236	3.0	130	223	1.7
	3	165	442	872	1.9	56	229	4.1	108	196	1.8
	3	82	648	2098	3.2	82	280	3.4	134	379	2.8
	4	165	418	1193	4.8	65	301	4.6	123	219	1.7
	4	82	638	1837	2.9	88	286	3.3	129	188	1.4

* In mg per kg injected on day 0.

spleen. This highest dose is 0.8 ml of a 30% spleen cell suspension and approaches the maximum intravenous dose that is tolerated by mice as a single injection. Experiments not included in this figure indicate that cyclophosphamide tolerance to sheep cells does not follow injection of ox or rabbit erythrocytes.

Response of Spleen DNA and RNA Synthesis to Cyclophosphamide.—Tables I and II present data from two experiments in which DNA and RNA synthesis following cyclophosphamide administration were studied. In Table I the actual data of quantity, rate of synthesis, and specific activity of DNA, nuclear

RNA, and cytoplasmic RNA are presented. Table II compares the synthetic rates to those of animals who have not received cyclophosphamide, and includes antibody titers from a parallel group of animals challenged with sheep cells on the day the rate of synthesis was determined. The point to be made is that in

TABLE II

Relative Rates of DNA and RNA Synthesis and Immunosuppression at Various Times after Cyclophosphamide

Experiment No.	Day	Cytoxan dose*	DNA synthesis‡	Nuclear RNA synthesis‡	Cytoplasmic RNA synthesis‡	Antibody titers§
1	0	—	1.00	1.00	1.00	10
	1	330	0.05	0.72	0.54	0
	2	330	0.07	0.69	0.89	0
	3	330	0.06	0.65	0.56	0.7
	4	330	0.17	0.84	0.94	8.7
	5	330	0.20	0.68	—	—
	6	330	0.94	0.59	1.32	9
2	0	—	1.00	1.00	1.00	11.3
	1	165	0.07	0.71	0.41	0
		82	0.52	1.00	0.65	12
	2	165	0.24	0.65	0.59	10
		82	0.35	0.97	0.53	12
	3	165	0.35	1.32	0.56	11
		82	0.59	1.10	0.91	12
	4	165	0.89	1.48	0.53	11
		82	0.54	1.06	0.44	11

* In mg per kg on day 0.

‡ Derived from Table I and expressed as ratio of specific activity to specific activity on day 0.

§ Average hemagglutination titer expressed as $\log_2/10$. Cyclophosphamide was given to separate groups of 3 animals and sheep cells injected on the day indicated. The animals were bled and titered 30 days later. The figures for nucleic acid synthesis were those existing at the time sheep cells were injected in these animals.

both Experiments I and II, best appreciated in Table II, profound suppression of immunological responsiveness correlated with profound depression of DNA synthesis at the time antigen is injected. Thus, complete immunological tolerance was associated with DNA-specific activities which were less than one-tenth those of the untreated spleen. No similar correlation of immune suppression with nuclear or cytoplasmic RNA synthesis is evident.

It will also be noted in Experiment I (Table I) that the amounts of DNA and nuclear RNA fall to about 20% of their precyclophosphamide levels, the maximum depression occurring on the 4th or 5th day after drug injection. However, the reduction of cytoplasmic RNA is much less, the minimum level of cytoplasmic RNA is still more than 40% of the predrug level.

TABLE III

Plaque-Forming Spleen Cells 4, 6, and 17 Days after Cyclophosphamide Administration which was Begun Various Times after the Injection of Sheep Erythrocytes

Day cytoxan begun*	Plaques on day cytoxan begun‡		Plaques 4 days after cytoxan begun		Plaques 6 days after cytoxan begun		Plaques 17 days after cytoxan begun	
Day 0								
19S plaques	120		1		1		3	
7S plaques		8		0		0		0
Day 2								
19S plaques	2800		3		4		3	
7S plaques		56		0		0		2
Day 3								
19S plaques	20,800		50		40		1900	
7S plaques		10,800		18		16		1260
Day 4								
19S plaques	50,000		52		39		520	
7S plaques		42,000		22		24		510
Day 14								
19S plaques	750		83		56		238	
7S plaques		5300		117		35		119
Day 28								
19S plaques	305				36		102	
7S plaques		1470				16		151
Day 46								
19S plaques	235							
7S plaques		550						

* 0.8 ml of 30% sheep cells injected day 0. 82 mg per kg of cyclophosphamide daily for 4 days. All results are expressed in plaques per spleen.

‡ These animals received no cyclophosphamide.

Analysis of Spleen Plaques when Cyclophosphamide is Begun at Varying Times after Sheep Cell Administration.—It was noted in Fig. 1 that a single dose of cyclophosphamide (330 mg per kg), given with sheep cells or 1 or 2 days after sheep cell injection, was associated with essentially complete immunological tolerance, while if the drug was delayed until the 3rd day there was virtually no induction of tolerance. In order to investigate those events taking place between days 2 and 3 which change drug sensitivity so remarkably, the alterations in antibody-forming cells of the spleen have been analyzed when cyclophospha-

mide is given after sheep cells on various days (Table III). Thus cyclophosphamide was given as a 4 day course (82 mg per kg per day) beginning the day of sheep cell injection, or beginning 2, 3, 4, 14, or 28 days thereafter. In each instance the 19S and 7S hemolysin-producing plaques of the spleen were determined on the day cyclophosphamide was begun and 4, 6, and 17 days later, each time point representing the spleens from three animals.

In Table III, the first two columns of data, scanned vertically, indicate the sequence of 19S and 7S antibody-forming cells following sheep cell challenge in the absence of immunosuppressive drug. The results of this experiment are representative of those we have seen. Thus, in our studies 19S cells appear in large numbers only on the 2nd day after sheep cell injection, reach a maximum on the 4th day, and decline rather rapidly over the ensuing week. Cells producing 7S antibody appear in significant numbers a day later (day 3) and decline much more slowly. It will be observed that between days 2 and 3, the period during which sensitivity to cyclophosphamide is lost, there are marked alterations in the number of both 19S and 7S plaque-forming cells: 19S cells increase 8-fold and 7S cells increase 200-fold.

For the sake of simplicity, data on the total number of spleen cells has not been included in the table. On the day cyclophosphamide was begun, the combined average spleen cell number was 156 million, on day 4 after cyclophosphamide the average was 36 million, on day 6 it was 32 million, and on day 17 it was 156 million. The period of observation included in the table encompasses the period of restoration of the total number of spleen cells.

The remaining columns of Table III list the 19S and 7S plaque-forming cells 4, 6, and 17 days after the initial drug injection. Thus, when cyclophosphamide is begun the day of sheep cells or 2 days later, there is complete disappearance of hemolysin-producing plaques by drug day 4 and continued absence on day 17. This continued absence of plaques confirms the antibody data that cyclophosphamide administered on these days produced complete immunological tolerance.

Cyclophosphamide administered 3 days or more after antigen injection, on the contrary, leaves a considerable number of both 19S and 7S plaque-forming cells 17 days after treatment, when the spleen is restored to its pretreatment cell number. When the drug is begun 3, 4, or 14 days after antigen there is a considerably greater depression of plaque-forming cells on days 4 and 6 post-drug, at the height of cyclophosphamide effect, than on postdrug day 17 when the spleen cell level has returned to normal. Indeed, it has been found that the same amount of cyclophosphamide (330 mg per kg) injected as a single dose (rather than 4 daily divided doses) results in no detectable plaque-forming cells 4 days after the drug has been administered, in those animals which had received antigen 3 and 4 days earlier. However, substantial numbers of plaque-forming cells are again present on the 17th postdrug day in these animals, indicating that complete tolerance was not induced.

DISCUSSION

The experiments reported here confirm the work of others (5, 6) on the effectiveness of cyclophosphamide as an immunosuppressive agent. The ability to completely suppress antibody against sheep cells (18), a strong antigenic stimulus, indicates a high order of effectiveness in the mouse. The finding that maximum immunosuppression is achieved when cyclophosphamide is injected in the 24 hr before antigen is also consistent with the work of other investigators employing irradiation (19) and some immunosuppressive drugs (20, 21), although with other drugs and antigens maximum depression has been obtained when the antigen is injected first (20, 21).

It was at first puzzling that a much higher dose of antigen was necessary for complete drug-induced immunological tolerance (6.4×10^9 sheep cells) than for the maximum antibody response (2×10^8 cell, references 8 and 13), since it is likely that the same receptor site is involved in both antibody formation and tolerance. The answer to this problem is immediately apparent from a study of the relationship of drug-induced tolerance to antigen dose (Fig. 2), where it is seen that with an injection of 2×10^8 sheep cells tolerance is about 90% complete. If the same receptor sites were involved in antibody formation, this response would be 90% of maximum, and available methods for measuring immune capacity (including the plaque technique) could not be expected to detect the difference between 90 and 100%. It is probable that the increment of sheep cells from 2×10^8 to 6.4×10^9 is necessary to reach some 10% of the receptor sites which are relatively sequestered from the antigen source.

Quantitative data derived from study of antibody-forming cells with the Jerne technique indicate that there are two components to cyclophosphamide-induced immunological suppression. The first is not specific immunological tolerance since it is independent of administered antigen; it is nonspecific immune suppression, presumably persisting lymphoid injury caused by the cytotoxic drug. This nonspecific immune injury accounts for a large fraction (two-thirds to five-sixths) of the total immunological suppression seen 18 days after the usual dose of cyclophosphamide which was employed in the present experiments. From an examination of the decrease of DNA (Table I, Experiment 1) and from the fall in spleen cell count after cyclophosphamide injection, it is evident that the fraction of cells destroyed by cyclophosphamide is quantitatively similar to the fraction of nonspecific immune suppression resulting from this dose of the drug. Thus the data suggest that this nonspecific immune suppression may be related to drug-induced cell death. The total number of lymphoid cells of the spleen are restored 18 days after the drug, but at this time they are defective in their immunological competence. (A further publication will describe the recovery of competence over ensuing months under thymic influence.) In reviewing the nucleic acid data (Table I) it is also of interest that there is a much greater loss of DNA and nuclear RNA than of cytoplasmic

RNA after cyclophosphamide, suggesting that the cells killed by the drug are poor in cytoplasm. The small lymphocyte is such a cell, poor in cytoplasm.

The second and remaining fraction of immunological suppression is specific immune tolerance; i.e., directed specifically towards sheep cells and requiring sheep cell administration at the time of drug injection. This immune tolerance induction of cyclophosphamide correlates well in the present experiments with profound suppression of DNA synthesis in the remaining lymphoid cells of the spleen (those not destroyed by cyclophosphamide). Thus, the nucleic acid evidence favors a mechanism for cyclophosphamide tolerance in which tolerance results from immunological stimulation in the presence of profound inhibition of DNA synthesis and mitosis (21–24). Further, it appears plausible that the immunological stimulation of such DNA-damaged cells leads to their death, with consequent elimination of the specific clone (25, 26). Since the primary site of action of cyclophosphamide is known to involve DNA (27), this explanation is quite acceptable in connection with this compound. It seems reasonable that other immunosuppressive compounds act in the same manner.

The present experiments do not support the alternate explanation that has been put forth for drug-induced tolerance, i.e. that these compounds act in conjunction with antigen to turn off the transcription of immunological information within the receptor or effector immune cell via a ribonucleic acid mechanism (4, 21). However, it must be pointed out that the present studies measure only average nuclear and cytoplasmic ribonucleic acid synthesis within the spleen; a decrease in synthesis of RNA within a particular cell line or within a particular chemical compartment could go undetected.

An earlier paper (28) describes the prompt and antigen-specific disappearance of hemolysin-forming plaques of unstimulated spleen produced when sheep erythrocytes and cyclophosphamide are administered at the same time (Table III). This disappearance supports a clonal selection theory of antibody synthesis (26, 27), and suggests that the 19S-producing cell of the resting spleen is related to the specific clone which responds to the antigen.

Less revealing has been the attempt to analyze the events in tolerance induction when the drug has been given after antigen. Unfortunately, there is a marked increase in both 19S (8-fold) and 7S (200-fold) cells between the 2nd postantigen day when complete tolerance with cyclophosphamide can still be realized, and the 3rd postantigen day when the drug is no longer effective. The information from our experiments suggests that the 19S cell of the 2nd postantigen day is much more sensitive to cyclophosphamide than either the 19S or the 7S cell of the 3rd day. With the passage of time after antigen (day 14 and day 28 postantigen), increasingly cyclophosphamide-resistant cells emerge, though the 19S cell appears to be more resistant than the 7S cell at these later times. Thus, particularly with respect to the 19S antibody-producing cell, our evidence suggests a progressive increase in resistance to cyclo-

phosphamide during the first 2 wk following antigen. This could be due either to a decrease in the mitotic rate and consequent loss of sensitivity to cyclophosphamide or to a differentiation to cells of less sensitivity. The present experiments are consistent with the work of Sahier and Schwartz who, using 6-mercaptopurine, found that the 7S response could be eliminated at a dosage which left the 19S response intact (29).

Two factors make analysis of postantigen drug treatment with the plaque technique difficult. The first is the observation that during the peak of drug effect there is an eclipse of detectable plaque-forming cells, with reappearance after the drug effect has dissipated. It follows that potential plaque-forming cells are present at the peak of cyclophosphamide action but are undetected by plaquing at that time. Such cells may not be functioning because of drug damage, but regardless of the cause, the inability to follow the cells through the entire period of drug action makes interpretation of the results ambiguous. The second point which makes the analysis difficult is the present uncertainty of the relationship of the 19S and 7S cells to each other.

SUMMARY

Complete immunological tolerance to sheep cells can be induced in mice when cyclophosphamide is injected together with sheep cells or up to 72 hr before or 48 hr after the antigen. As is true for radiation-induced immune suppression, the drug is most effective when given in the 24 hr prior to antigen. Complete cyclophosphamide-induced immunological suppression requires large doses of sheep cells (6.2×10^9 cells), presumably to enable antigen to reach sequestered receptor sites.

The cyclophosphamide tolerance system has been analyzed with the Jerne technique to determine plaque-forming cells and with isotopic methods to measure rates of nucleic acid synthesis. This drug suppression has been found to consist of two components. The first is nonspecific injury to the lymphoid system caused by the cytotoxic drug and is related to the proportion of spleen cells killed. The second is antigen-specific immunological tolerance and appears to correlate with profound depression of deoxyribonucleic acid synthesis in the surviving cells. This tolerance is thought to be most consistent with a mechanism in which antigenic stimulation in the presence of cyclophosphamide-inhibited DNA synthesis and mitosis leads to the elimination or death of the specific immunological clone.

Tolerance induction with cyclophosphamide is associated with loss of the 19S hemolysin plaques which are seen in nonstimulated mouse spleen, implicating these cells in immune responsiveness. The ability to induce tolerance is lost on the 3rd postantigen day at the end of a 24-hr period in which 19S cells have increased 8-fold and 7S cells 200-fold. The data suggest that loss of sensitivity is due to the emergence on day 3 of drug-resistant plaque-forming cells, particu-

larly those of the 19S variety. In the succeeding days after antigen injection there is a progressive increase in the resistance of plaque-forming cells to cyclophosphamide administration.

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