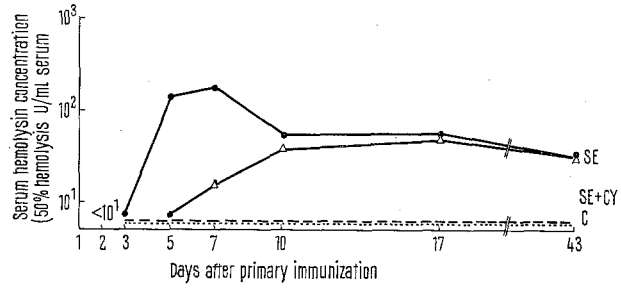
The Influence of Cyclophosphamide on the Process of Priming for the Secondary Response

The phenomenon of specific secondary responsiveness remains an incompletely understood problem in immunology. The findings of LEDUC, COONS and CONNOLLY[1] indicated that active antibody synthesis is not a prerequisite for immunological memory. This is in good agreement with the results of serological investigations showing that the secondary response was not inhibited at all, if the primary response was suppressed by 6-mercaptopurine[2] or chloramphenicol[3]. On the other hand, the process of priming was prevented by chloramphenicol in the serological studies of CRUCHAUD and COONS[4]. On the basis of the *X-Y-Z* scheme[2,5] the preparation for the secondary response depends on the persistence of *Y* or memory cells, which are the result of the specific action of the antigen on the *X* cells. According to NOSSAL, AUSTIN and ADA[6] memory cell production may develop concurrently with, but independently of, the cellular proliferative events leading to antibody formation. But it has been pointed out by SERCARZ and BYERS[7] that the transformation of *X* cells into *Y* cells requires the synthesis of IgM. Thus it was of interest to find out, at the cellular and humoral level, whether the inhibition of the primary response by treatment with a cytotoxic and alkylating agent such as cyclophosphamide (CY) prevents the preparation of the lymphoreticular tissue of mice for the secondary response.

Adult male mice of the inbred strain NMRI weighing 18–24 g were immunized i.p. with 4×10^8 sheep erythrocytes (SE) (group I). A second group of mice (group II) was treated by additional i.p. injection of CY (Endoxan, Asta, Brackwede/Germany) as a freshly made saline solution containing 4 mg/ml. A total of 8 mg CY was given per animal as 4 daily divided doses starting 2 days before immunization; 43 days after the primary antigenic stimulus a second dose of 4×10^8 SE was injected i.p. into the animals of both groups. At different intervals after the primary and secondary immunization, 6 mice out of each group and 2 animals of the corresponding controls were sacrificed, their spleens removed aseptically and their sera collected. For the quantitative determination of plaque-forming cells (PFC) the direct[8] and indirect technique[9] were employed. Suspensions of spleen cells in agarose (Behring-Werke Marburg/Germany) were poured out onto Oxoid No. 3 agar/DEAE dextran underlayers in petri dishes as described elsewhere[10,11]. Serum hemolysin activity of pooled serum samples from the controls and 6 identically treated mice was determined spectrophotometrically at 530 nm according to the 50% hemolysis method[12]. Hemolysin concentrations are given in 50% hemolysis units per ml of serum. Additionally those fractions of the total hemolysin activity were determined which are resistant to treatment by 0.125 *M* 2-mercaptoethanol (2-ME). The lowest values considered significant were ten 50% hemolysis units[13].

In the spleens of mice immunized with 4×10^8 SE (group I), the peak values of direct and indirect PFC were found 5 days after primary immunization. In contrast, in the CY-treated group (group II) the maximum values were reached 17 days after primary immunization and amounted only to 5% of either the direct (i.e. 90 direct PFC/10^6 spleen cells) or the indirect PFC (i.e., 247 indirect PFC/10^6 spleen cells) of the corresponding controls at day 5. Similar results were obtained in previous studies[14]. As can be seen from the Figure, essential amounts of serum hemolysins (i.e. $\geqq$ ten 50% hemolysis units) were not demonstrable in the sera of CY-treated mice during the 43 days of observation after primary immunization.

As compared with the primary response, the booster injection of 4×10^8 SE into the mice of group I resulted in a markedly reduced formation of direct PFC representing less than 25% of the peak value of the primary response (Table). Similar numbers were found in the spleens of CY-treated mice (Table, group II). Furthermore, it can be seen from the table that, simultaneously with the development of direct PFC, an increase of the numbers of indirect PFC was demonstrable, reaching a peak value 4 days after the secondary immunization. In the group I, at this time, about 1.15% of all spleen cells were engaged in hemolysin (7S) production, and in the spleens of CY-treated mice (group II) a peak value of 0.5% could be detected. This means that the inhibition of the primary response by treatment with CY does not actually prevent the unknown process of priming for the secondary response. The reduced secondary reaction of CY-treated mice is apparently due to the cytotoxic effect of the agent, because the injection of high doses of CY results in the decrease of the spleen weights to about 50%, characterized by a nearly complete loss of the red pulp[15].



Suppression of the hemolysin production by treatment with cyclophosphamide (CY) after the primary immunization with 4×10^8 sheep erythrocytes (SE). C, untreated control. Hemolysin concentration is given in 50% hemolysis units/ml of serum. ●, total hemolysin activity; △, fractions of the total hemolysin activity resistant to 2-mercaptoethanol.

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Effect of an initial treatment with cyclophosphamide on the development of antibody-forming spleen cells during the secondary immune response

Days after second antigen injection	Average numbers of plaque-forming cells/10^6 spleen cells in the groups:									
	Group I				Group II					
	$\bar{X}$DPFC[a]	$\pm s$[b]	$\bar{X}$IPFC[c]	$\pm s$[b]	$\bar{X}$DPFC[a]	$\pm s$[b]	P[d]	$\bar{X}$IPFC[c]	$\pm s$[b]	P[d]
0[e]	6	4.2	97	49.2	1	0.8	> 0.1	19	5.4	> 0.1
2	21	7.7	378	107.7	38	7.5	> 0.1	85	15.8	< 0.05
3	468	145.6	3762	703.1	276	110.6	> 0.1	827	271.7	< 0.05
4	316	123.7	11497	2539.1	112	18.9	> 0.1	4960	112.4	< 0.05
5	148	50.2	5840	2398.6	62	24.0	> 0.1	1958	760.9	> 0.1
7	13	3.1	785	162.2	105	32.3	< 0.05	1058	267.8	> 0.1
10	34	9.1	1347	475.6	60	18.7	> 0.1	729	323.5	> 0.1
14	24	6.4	592	226.6	33	4.8	> 0.1	359	103.1	> 0.1
21	28	10.2	399	82.4	125	71.0	> 0.1	552	199.4	> 0.1
28	16	3.9	304	71.8	8	3.0	> 0.1	129	36.5	< 0.05
35	15	2.3	534	212.9	23	6.1	> 0.1	243	60.7	> 0.1

[a] Mean value of direct plaque-forming cells; [b] standard deviation; [c] mean value of indirect plaque-forming cells; [d] statistical significance evaluated according to the Student's t-test; [e] 43 days after primary immunization.

Contrary to the situation after primary immunization, the majority of the serum hemolysins determined after the second antigen injection was resistant to treatment with 2-ME. In the sera of CY-treated mice (group II), the average peak titre of 7S hemolysins determined 10 days after secondary immunization was 132 50% hemolysis units. This value represented 60% of the peak value of the corresponding control (group I) found 7 days after the secondary antigenic stimulus.

In consequence it may be concluded that memory cells can develop after primary immunization without being actively engaged in antibody synthesis.

Zusammenfassung. Wurde die primäre Immunitätsreaktion von mit 4×10^8 Schaferythrozyten immunisierten Mäusen durch Cyclophosphamid unterdrückt, fanden sich während der 43tägigen Beobachtungszeit keine nennenswerten Serumhämolysin- und Agglutinintiter, und in der Milz solcher Tiere konnte im Vergleich zur Kontrolle nur jeweils 5% der direkt und indirekt Plaques bildenden Milzzellen nachgewiesen werden. Dennoch führte die Zweitimmunisierung zu einer typischen Sekundärreaktion.

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Media-Dependent Antagonism of Gentamicin Sulfate by Liquoid (Sodium Polyanetholsulfonate)

Recently we reported that sodium polyanetholsulfonate (Liquoid®) antagonizes the activity of both kanamycin sulfate and polymyxin B in vitro[1], confirming earlier findings by other investigators with regard to streptomycin and polymyxin B[2,3]. Meanwhile we have extended our studies to several additional antibiotics. Neither carbenicillin, lincomycin, nor amphotericin B were affected by Liquoid in vitro, using a variety of broth media[1], in the absence or presence of fresh and heat-inactivated human serum. However, as was to be expected, Liquoid diminished the activity of gentamicin sulfate (Schering Corp., Bloomfield, N.J.) markedly in nutrient broth (NB), but much less so or not at all in Mueller-Hinton (MHB), trypticase soy (TSB), and thioglycollate (TGCB) broth. In contrast to our earlier findings with regard to kanamycin sulfate and polymyxin B, 10% fresh serum did not enhance the antagonistic effect of Liquoid versus gentamicin. The addition of Liquoid (0.05%) to assay tubes containing serial twofold dilutions of gentamicin in 10% fresh serum, regardless of the broth employed, gave rise to a faint turbidity which increased in intensity over several hours' incubation at 37°C, thus rendering the minimal inhibitory concentration (MIC) readings rather difficult. This turbidity phenomenon prompted us to determine the minimal bactericidal concentration (MBC) of gentamicin versus strains of *Escherichia coli* and *Pseudomonas aeruginosa* (the assay tubes contained 1.5×10^6 organisms/ml at 0 time). As shown in the Table, the MIC values obtained demonstrate that the antagonistic effect of Liquoid against gentamicin is media-dependent (NB) and certainly not enhanced in the presence of 10% fresh serum.

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