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Cyclic Development of Immunological Memory to Bacterial Lipopolysaccharide

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Immunological memory to the lipopolysaccharide of *Escherichia coli* O113 was generated in strains of inbred mice given a single subimmunogenic dose of either *E. coli* O113 lipopolysaccharide or the native protoplasmic polysaccharide of *E. coli* O113. Such memory, which only involved antibody of the immunoglobulin M class, developed in a cyclic manner that was characteristic for the strain of mice used. It involved cell proliferation as well as differentiation and persisted for at least 25 days after priming with a single injection of a subimmunogenic dose of *E. coli* O113 lipopolysaccharide.

Two signals are required for the development and expression of a secondary antibody response (immunological memory) to the lipopolysaccharide (LPS) of *Escherichia coli* O113 (LPS O113). One signal is antigen specific and can be elicited by the nonmitogenic native protoplasmic polysaccharide (NPP) of *E. coli* O113 (NPP O113); NPP O113 possesses the O antigenic determinants of LPS O113 (29). The second signal is mediated by the lipid-A portion of the LPS molecule. Both NPP O113 and LPS O113 can prepare (prime) mice to give a secondary antibody response; however, only LPS O113 can "trigger" the expression of memory in mice primed with either NPP O113 or LPS O113 (29).

In the present work, long-term kinetic studies were conducted to better define the separate events or processes involved in the development of memory in mice primed with a single subimmunogenic dose of LPS O113. The results show that the generation of memory proceeds in a cyclic manner and involves both cell proliferation and differentiation. Strains of inbred mice showed quantitative, as well as qualitative, differences in the cyclic pattern expressed; this suggests that some of the events associated with the cyclic pattern may be under genetic control.

MATERIALS AND METHODS

Animals. Female BALB/cCum mice were obtained from Cumberland View Farms, Clinton, Tenn., and female AKR/N mice were obtained from the Small Animal Section, Veterinary Resources Branch, Division of Research Services, National Institutes of Health, Bethesda, Md. All mice were 8 to 10 weeks old at the time of use.

Antigens. LPS was extracted from cell walls of *E. coli* O113 (Braude) by the phenol-water procedure (30). The immunological properties of this preparation of LPS (LPS O113) have been described in detail (14, 23).

NPP, which is free of lipid-A as well as lipid-A-associated protein and thus contains only the polysaccharide determinants of LPS O113 (2, 3, 24), was extracted with cold trichloroacetic acid (3) from the protoplasmic fraction of *E. coli* O113 (Braude). Mice were injected intraperitoneally (i.p.) with known amounts of either LPS O113 or NPP O113 in 0.2 ml of saline as stated below.

Immunological methods. Antibody-producing plaque-forming cells (PFC) making IgM antibody specific for LPS O113 were detected by a slide version of the technique of localized hemolysis-in-gel, with sheep erythrocytes sensitized with LPS O113 as indicator cells (14). The results obtained for individual mice (PFC per spleen) were corrected by subtraction for the small number of sheep erythrocyte-specific background PFC found, so that only values for LPS O113-specific PFC are considered in this report. In all cases, the magnitude of the PFC response was determined at peak, i.e., 4 days after immunization with an optimally immunogenic dose (10 to 20 μg) of LPS O113 (14, 23).

Two experimental approaches were used to measure the degree of immunological memory generated in mice after pretreatment (priming) with a single i.p. injection of different amounts of either LPS O113 or NPP O113. In the first approach, values for PFC per spleen for individual primed mice immunized with 10 μg of LPS O113 were divided by the mean number of PFC per spleen detected for groups of 8 to 10 unprimed mice immunized with 10 μg of LPS O113 (unprimed controls); next, the mean relative increase in the PFC response due to priming (± the standard error of the mean) was calculated for groups of 8 to 10 similarly treated mice. In the second experimental approach, the memory factor (MF) was used to provide a more precise measurement of the degree of memory present at different times after priming (20, 26). For example, in the case of the day 4 PFC response of mice immunized with 10 μg of LPS O113, 8 days after priming with a single injection of LPS O113, the MF for a single immunized primed mouse was calculated as follows: $MF = PFC(0, 8) - PFC_{\bar{x}}(0)/PFC_{\bar{x}}(8)$, where PFC(0, 8) is PFC per spleen for a single mouse immunized 8 days after priming (day 0); $PFC_{\bar{x}}(0)$ is the mean number of PFC per spleen for a group of 8 to 10 primed mice not immunized with LPS O113; and $PFC_{\bar{x}}(8)$ is the mean number of PFC per spleen for a group of 8 to 10 unprimed mice immunized with LPS O113. The data then were expressed as the mean MF ± the standard error of the mean for groups of 8 to 10 similarly treated mice. It should be noted that values for $PFC_{\bar{x}}(0)$ were negligible (<50 PFC per spleen) for mice primed with 0.005 μg (or less) of LPS O113 or NPP O113; consequently, when such low priming doses were used, values for the mean MF equaled those for the mean increase in the PFC response due to priming. Since values for PFC per spleen are log-normally distributed (13), data for $PFC_{\bar{x}}(8)$ and other terms of the

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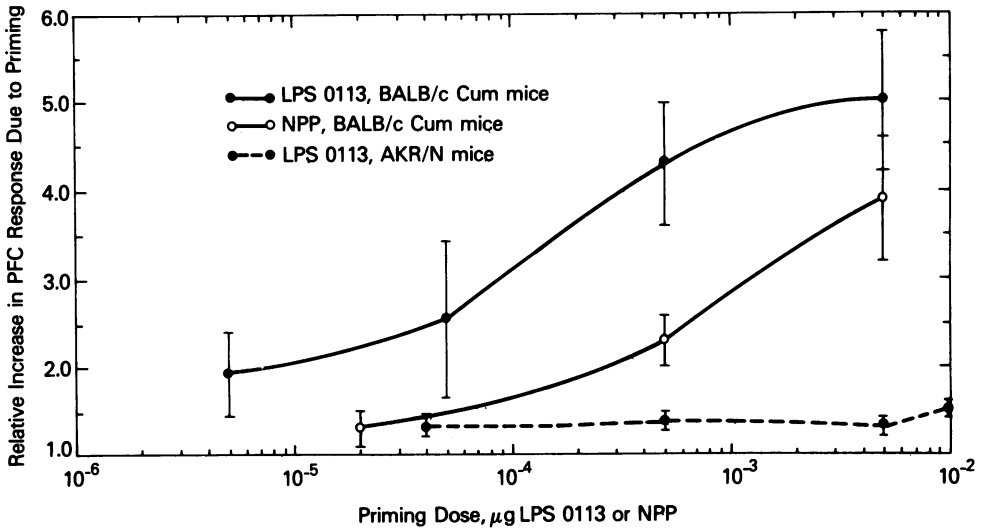

FIG. 1. Effect of priming dose of LPS O113 or NPP O113 on the development of immunological memory to LPS O113 in BALB/cCum and AKR/N mice.

equation were expressed as the geometric mean (antilog) of values for the mean $\log_{10}$ PFC per spleen for these groups of mice. Since the main focus of this work is in the degree of memory generated after priming with LPS O113, as measured by calculated values for the MF, actual values for mean number of PFC per spleen are not shown for the sake of brevity. However, these values can be determined from the MF and control values given for the experiments discussed.

Statistics. Student's *t*-test was used to evaluate the significance of the differences observed. Differences were considered to be significant when P values of <0.05 were obtained.

Mitotic inhibitor. Vinblastine sulfate (Velban), a mitotic inhibitor (21, 28), was purchased from Eli Lilly and Co., Indianapolis, Ind. Mice were given 20 μg of vinblastine sulfate i.p. in 0.5 ml of saline at stated times after priming; next, the effect of treatment with vinblastine sulfate on the generation of memory was assessed by changes in the calculated values for the MF at each of the times considered.

RESULTS

Minimal dose of LPS O113 or NPP O113 required for the generation of memory. Groups of 10 mice were given a single i.p. injection of a subimmunogenic dose (5×10^{-6} μg to 5×10^{-3} μg) of either LPS O113 of NPP O113. After 8 days, they were immunized (i.p.) with 10 μg of LPS O113, and the magnitude of the LPS O113-specific PFC response produced was determined 4 days after immunization. The results obtained were compared with those of unprimed mice immunized with 10 μg of LPS O113 to calculate the mean relative increase in the magnitude of the PFC response due to priming.

Significant ($P < 0.05$) immunological memory, which consisted of more than a 1.4-fold increase in the magnitude of the PFC response to 10 μg of LPS O113, was generated in BALB/cCum mice primed with as little as 5×10^{-5} μg of LPS O113 (Fig. 1). Larger priming doses resulted in the development of increased memory. Significant memory was generated in BALB/cCum mice primed with NPP O113, a finding consistent with the results of previous studies with other strains of mice (29); however, about 100 times more NPP O113 than LPS O113 was needed to generate the same degree of memory. By contrast, only a minimal degree of memory was generated in AKR/N mice primed with the same amount of LPS O113 (Fig. 1).

Since the data of Fig. 1 relate solely to the effects of priming on the magnitude of the IgM antibody response to LPS O113, other studies were conducted to determine whether both BALB/cCum and AKR/N mice primed 8 days earlier with 0.005 μg or more of LPS O113 also make antibodies of the γ_1, γ_{2a}, γ_{2b}, and γ_3 class on 4, 8, or 21 days after immunization with 10 μg of LPS O113. PFC-making LPS O113-specific antibodies of these isotypes were not detected (data not shown). Thus, the memory generated under the experimental conditions described (Fig. 1) involves only cells making antibody of the IgM class.

Additional attempts to elicit a secondary antibody response to LPS O113 in AKR/N mice. Since only a minimal degree of memory, at best, was generated in AKR/N mice 8 days after priming with 10^{-4} to 10^{-2} μg of LPS O113 (Fig. 1), an attempt was made to determine whether greater memory could be generated in mice primed with larger doses of LPS O113. Groups of AKR/N mice were primed with a single i.p. injection of 0.01, 1, or 20 μg of LPS O113; 21 days later, they were immunized i.p. with 10 μg of LPS O113, and the magnitude of the PFC response produced 4 days after immunization was compared with that of unprimed mice to determine whether priming with these large doses of LPS O113 generated the development of increased memory.

Priming with the doses of LPS O113 used did not augment the capacity of AKR/N mice to make an antibody response to LPS O113 ($P > 0.05$ for all comparisons to unprimed controls); this was the case for data expressed as PFC per spleen and as PFC per 10^6 nucleated spleen cells (Table 1). Furthermore, priming with three weekly injections (i.p.) of LPS O113 did not result in the development of memory ($P > 0.05$). Since these experimental approaches were found to be effective in eliciting the development of significant memory in other strains of inbred mice (15), the results indicate that only a modest degree of memory, at best, can be generated to LPS O113 in AKR/N mice. In comparison with BALB/c mice, unprimed AKR/N mice usually gave a lower PFC response to 10 to 20 μg of LPS O113 (Table 1; reference 4).

Kinetics for the generation of immunological memory. Groups of 10 BALB/cCum mice were primed with a single i.p. injection of a subimmunogenic dose (0.005 μg) of LPS

O113; 3 to 25 days after priming, they were immunized (i.p.) with a single injection of 10 μg of LPS O113, and the magnitude of the LPS O113-specific PFC response produced was determined 4 days after immunization. The results obtained were compared with those for a group of 8 to 10 unprimed immunized mice, as well as for a group of 8 to 10 primed unimmunized mice (control group), so that the MF could be calculated for each time considered. This enabled us to quantitate the degree of immunological memory generated with time after priming. Separate control groups were used for each time considered; thus, the resultant MF value was based on calculations involving more than 20 mice. More than 500 mice were used in the entire study summarized in Fig. 2.

Memory, as measured by significant ($P < 0.05$) increases in mean MF values, developed in a cyclic manner for BALB/cCum mice primed with a single subimmunogenic dose of LPS O113 (Fig. 2). Three or four distinct memory peaks were evident; these occurred at 8, 12, 21, and 24 days after priming. Significant ($P < 0.05$) decreases in memory were noted between days 8 to 9 (61% decrease), 12 to 14 (67% decrease), and 24 to 25 (43% decrease) after priming; however, after such decreases, the resulting MF values still were large enough (>1.4) to indicate that a significant ($P < 0.05$) degree of memory remained at the end of these times. Although memory appeared to increase at a similar rate between days 4 to 8 (4.5 fold) and days 9 to 12 (2.9 fold) after priming, the development of memory was much more rapid and prolonged between days 14 to 24 (15.8 fold) after priming. This resulted in extremely large MF values at 21 and 24 days after priming, the time at which memory was found to be maximal in other studies (29). Greater variation was noted in the degree of memory generated between days 20 to 24 after priming; this was probably due to the fact that memory was increasing so rapidly during this interval. Nevertheless, the large MF values obtained were reproducible and consistent with the results of other studies (29).

When the same type of study was conducted using low-responding AKR/N mice, a different kinetic pattern was obtained (Fig. 3). Here, significant memory was demonstrable at 8, 11, 14, and 18 to 20 days after priming with a single injection of 0.005 μg of LPS O113. Although the results suggest a cyclic pattern for the development of memory, the maximal degree of memory achieved in these mice was relatively modest (no more than two- to threefold at best) and far below that noted for BALB/cCum mice at corresponding times after priming (Fig. 2).

Contribution of cell proliferation to the generation of memory to LPS O113. Groups of 10 BALB/cCum mice were primed with a single i.p. injection of 0.005 μg of LPS O113 and were given a single i.p. injection of 20 μg of vinblastine sulfate on days 3 to 6 after priming, i.e., during the time memory was increasing at a steady rate (Fig. 2). All mice were immunized i.p. with 2 μg of LPS O113 8 days after priming, and the magnitude of the PFC response produced was determined 4 days after immunization. Mice were immunized with 2 μg rather than 10 μg of LPS O113 to reduce the combined toxic effects of both LPS and vinblastine sulfate. The effect of treatment with vinblastine sulfate on the generation of memory was evaluated with respect to its influence on the MF values obtained for each time considered.

Treatment with vinblastine sulfate, a mitotic inhibitor, caused a significant ($P < 0.05$) decrease in the development of memory (MF value) when given on days 3 and 4 after priming with 0.005 μg of LPS O113 (Table 2), i.e., during the first peak in the cyclic development of memory for LPS O113 in BALB/cCum mice (Fig. 2). Since treatment with vinblastine sulfate did not influence the magnitude of the PFC response of unprimed mice when given at corresponding times before immunization with 2 μg of LPS O113 (data not shown), these findings indicate that cell proliferation plays a significant role in the generation of a pool of memory

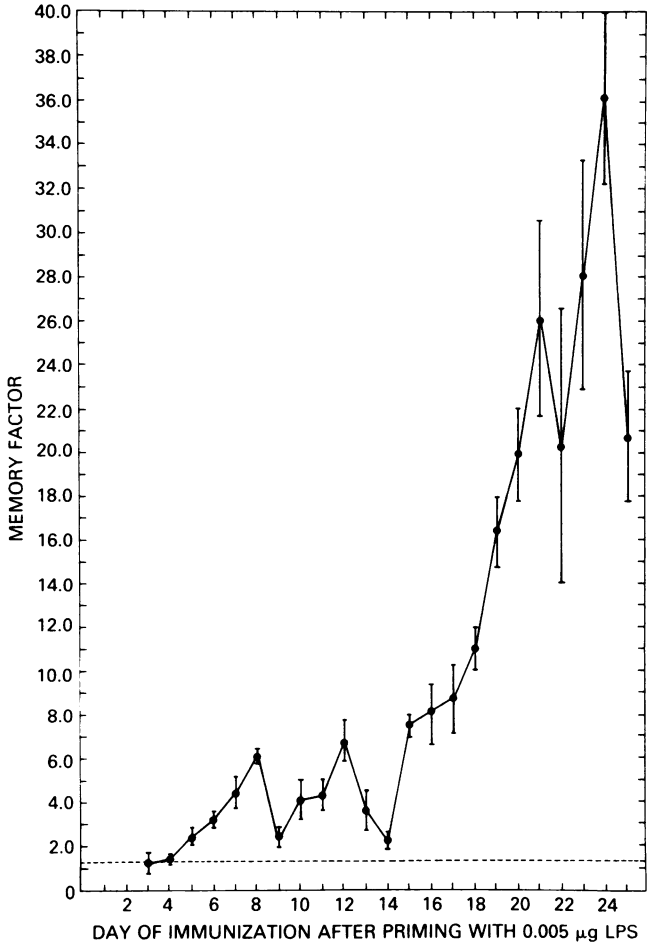

FIG. 2. Effect of priming (i.p.) with 0.005 μg of LPS O113 on the expression of immunological memory in BALB/cCum mice immunized i.p. with 10 μg of LPS O113. The broken horizontal line represents the MF value (1.4), above which the results obtained differ significantly ($P < 0.05$) from those for unprimed controls. In this study, $PFC_{\bar{x}}(0) = <50$, and $PFC_{\bar{x}}(8) = 7{,}319$.

TABLE 1. Inability of AKR/N mice to give a more profound secondary response to LPS O113 after priming with larger amounts of LPS O113

Immunization (μg of LPS) schedule (day)				PFC per spleen[a]	PFC per 10^6 spleen cells[b]
0	7	14	21		
			10	3.360 ± 0.079 (2,293)	1.301 ± 0.092 (20.0)
20			10	3.706 ± 0.141 (5,078)	1.237 ± 0.238 (17.2)
1			10	3.398 ± 0.093 (2,499)	1.338 ± 0.085 (21.8)
0.01			10	3.511 ± 0.118 (3,243)	1.411 ± 0.126 (25.7)
20	10	10	10	3.356 ± 0.129 (2,268)	1.213 ± 0.124 (16.3)

[a] Log_{10} PFC per spleen ± standard error of the mean for five mice 4 days after the last injection (i.p.) of LPS O113; geometric means are in parenthesis.

[b] Log_{10} PFC per 10^6 nucleated spleen cells for five mice 4 days after the last injection (i.p.) of LPS O113; geometric means are in parenthesis.

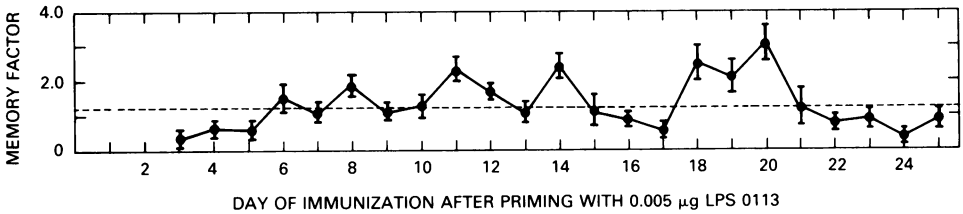

FIG. 3. Effect of priming (i.p.) with 0.005 μg of LPS O113 on the expression of immunological memory in AKR/N mice immunized i.p. with 10 μg of LPS O113. The broken horizontal line represents the MF value (1.4), above which the results obtained differ significantly ($P < 0.05$) from those for unprimed controls. In this study, $PFC_{\bar{x}}(0) = <50$, and $PFC_{\bar{x}}(8) = 2,400$.

B cells after priming with a subimmunogenic dose (0.005 μg of LPS O113.

It should be noted that the degree of memory generated in primed mice immunized with 2 μg of LPS O113 (17 to 22-fold; Table 2) appeared to be greater than that produced in primed mice immunized with 10 μg of LPS O113 (sixfold; Fig. 1). Although the same dose of LPS O113 was used for priming in both cases, unprimed BALB/c mice immunized with 2 μg of LPS O113 produce fewer PFC per spleen than unprimed BALB/c mice immunized with 10 μg of LPS O113 (4,550 ± 503 versus 7,319 ± 1,330 PFC per spleen, respectively). The former would provide a lower denominator in the equation used for the calculation of the MF; this would result in a higher MF value.

DISCUSSION

The development of immunological memory to LPS O113 has several prominent characteristics. In contrast to the results usually obtained with other types of antigens (reviewed in reference 12), neither the induction nor the expression of memory to LPS O113 is T-cell dependent; the degree of memory generated in athymic mice is essentially the same as that produced in genetically related thymus-bearing controls (29). Although the expression of immunological memory to helper T-cell-dependent antigens involves the synthesis of antibody mainly of the IgG class (4, 19), only IgM antibody was detected during a secondary antibody response to LPS O113; this is true for mice primed with either a large dose (10 μg) or a subimmunogenic dose (0.005 μg) of LPS O113 (14; this work). Previous studies have shown that two distinct signals are required for the induction and expression of memory to LPS O113 (29). One signal is antigen specific and can be delivered by subimmunogenic amounts of either NPP O113 or LPS O113, both of which possess the same O antigenic determinants (2, 3, 24, 29). The net result of this signal is the expansion of a pool of memory cells. Such expansion, which also appears to occur in mice primed with a subimmunogenic amount of other antigens (1, 9, 11, 16–18, 22, 26), occurs in the absence of detectable serum antibody or antigen-specific PFC or both and is evident only when mice are given a second injection of antigen; it must involve cell proliferation as well as differentiation, since it can be reduced substantially by treatment with vinblastine sulfate, an antimitotic agent (Table 2). The second signal involved in the expression of memory to LPS O113 is required for the activation of the expanded pool of memory cells generated after prior exposure to NPP O113 or LPS O113; it is nonspecific in nature and can be delivered by the lipid-A fraction of the LPS molecule (29). To be effective in expressing memory, both signal inducers, i.e., the O antigenic determinants and the lipid-A component, must be bound to the same molecule (29). Consequently, only LPS O113 can elicit the expression of a secondary antibody response in mice primed with either NPP O113 or LPS O113 (29). Although significant memory was generated in BALB/cCum mice primed with NPP O113, about 100 times more NPP O113 than LPS O113 was needed to generate the same degree of memory, a finding for which we have no complete explanation. Differences in molecular size and epitope density certainly could influence the interaction between precursors of memory cells and NPP O113 or LPS O113; this would result in differences in the size of the memory cell pool generated in response to either priming agent. Alternatively, NPP O113 and LPS O113 may be quite similar, but not entirely identical, in structure; thus, some of the differences noted with respect to the degree of memory generated could be due to the expansion of cross-reactive clones of memory cells. Obviously, more information is needed before we can decide between these and other possibilities.

TABLE 2. Effect of treatment with vinblastine sulfate on the generation of memory to LPS O113

Vinblastine sulfate administration (day)[a]	MF value ± SEM[b]	P value[c]
+3	5.3 ± 1.3	<0.05
+4	12.1 ± 1.8	<0.05
+5	17.7 ± 2.4	>0.05
+6	22.4 ± 2.5	>0.05

[a] Groups of 10 BALB/cCum mice were given a single i.p. injection of 20 μg of vinblastine sulfate at 3 to 6 days after priming (i.p.) with 0.005 μg of LPS O113 (day 0).

[b] Mice were immunized i.p. with 2 μg of LPS O113 8 days after priming, and the magnitude of the PFC response produced was determined 4 days after immunization. The mean number (± standard error of the mean) of PFC per spleen for unprimed mice immunized with 2 μg of LPS O113 was 4,550 ± 503.

[c] Based on comparisons to controls, i.e., primed and immunized mice not given vinblastine sulfate.

The most striking feature associated with the development of memory to LPS O113 is the cyclic pattern generated in mice primed with a single subimmunogenic dose of this antigen (Fig. 2 and 3). This suggests the involvement of an ordered regulatory process which, because of differences in the kinetic patterns expressed by inbred strains of mice, may be under separate genetic control. In this context, there is evidence to indicate that the generation of functional B-memory lymphocytes specific for trinitrophenylated LPS, a thymus-independent antigen, may be linked to genes coding for the variable region of immunoglobulin heavy chains (10). Since the development of memory to other antigens has not been monitored at closely spaced intervals over a prolonged period of time, as was done in the present work, one can not assert that the cyclic pattern observed is unique for LPS O113. A cyclic pattern has been reported to occur in an in vitro model for the expansion of dinitrophenyl-reactive memory cells (5); however, in contrast to the present work

in which memory was generated in response to a subimmunogenic dose of antigen in the absence of detectable antibody, the cyclic development of dinitrohenyl-reactive memory cells was found to be dependent upon the presence of excess persistent antigen and biofeedback inhibition due the interaction of antigen-antibody complexes with cell-surface immunoglobulin (5, 6). Furthermore, in contrast to the present work in which memory was both generated and expressed in the same intact animal, most other studies of memory rely on the results of adoptive cell-transfer experiments; the latter experimental approach could interfere with the continuous recruitment of memory cells by residual antigen or the expression of homeostatic regulatory mechanisms or both (reviewed in references 7 and 8). Obviously, in the case of other antigens, IgG-mediated suppression might be expected to alter the kinetics for the expression of IgM antibody formation and memory (8). Indeed, the complexities associated with these issues, as well as inherent differences with regard to immunogenicity and dose-response relationships for particular antigens, make it extremely difficult to compare, or to generalize from, the results of the variety of experimental models used for the generation and expression of memory. Although the results of the present work suggest that the development of memory to LPS O113, in conjunction with restimulation by small amounts of residual antigen, could play a role in the expression of a cyclic antibody response after primary immunization with an optimally immunogenic dose (10 to 20 μg) of LPS O113 (14), there may not be a direct relationship between these cyclic phenomena; it is possible to elicit a cyclic antibody response to LPS O113 in C3H/HeJ mice (14), which lack the ability to make a secondary antibody response to this antigen (25). The results of other studies on the expression of a cyclic antibody response after primary immunization with LPS have been discussed in great detail elsewhere (14) and will not be reviewed again in this report.

At this time, we have no complete explanation for the rather steep declines in memory noted between days 8 to 9 (61% decrease), days 12 to 14 (67% decrease), and days 24 to 25 (43% decrease) after priming (Fig. 2). The magnitude of such declines suggests half-life values of 12 to 14 h, much less than the values of 15 to 100 days reported for the half-life of memory cells for other antigens (reviewed in reference 8). These declines contribute significantly to the development of a cyclic pattern, rather than a continued and prolonged (stepwise) increase, in the degree of memory generated with time after priming. They suggest the involvement of an active inhibitory or cytotoxic process for controlling the size of clones or subsets of memory cells specific for LPS O113. Since BALB/cByJ and AKR/N mice differ greatly in both the degree of memory expressed and its kinetic (cyclic) pattern, these regulatory mechanisms, as well as the ability to generate memory per se, may be under genetic control. These issues are being examined in current studies.

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