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Immunology

# Reaction of germinal centers in the T-cell-independent response to the bacterial polysaccharide α(1→6)dextran

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**ABSTRACT** **Primary immunization of BALB/c mice with α(1→6)dextran (DEX), a native bacterial polysaccharide, induces an unexpected pattern of splenic B-cell responses. After a peak of antibody-secreting B-cell response at day 4, deposition of dextran–anti-dextran immune complexes, as revealed by staining with both dextran and antibodies to dextran, occurs and persists in splenic follicles until at least the fourth week after immunization. Antigen-specific B cells appear and proliferate in such follicles, leading by day 11 to development of DEX-specific germinal centers as characterized by the presence of distinct regions of DEX$^+$ peanut agglutinin-positive (PNA$^+$) cells. At this time, fluorescence-activated cell sorter analysis also reveals the appearance of a distinct population of DEX$^+$ PNA$^+$ splenic B cells. In contrast, DEX$^+$ PNA$^-$ cells, characterized by intense cytoplasmic staining, are present outside of splenic follicles, peak at day 4 to day 5, and persist until at least day 28. The frequency of these cells correlates with DEX-specific antibody-secreting cells, as detected by the ELISA-spot assay. Thus, in addition to the expected plasma cellular response, the typical T-cell-independent type II antigen, DEX, surprisingly also elicits the formation of antigen-specific germinal centers. These observations raise fundamental questions about the roles of germinal centers in T-cell-independent immune responses.**

Germinal centers (GCs) arise transiently within primary follicles of lymphoid tissues after antigenic stimulation. Recent studies focusing on the roles of GCs in T-cell-dependent (TD) immune responses indicated that many of the critical events involved in B-cell activation and differentiation can occur in GCs, including clonal selection and expansion of B memory cells, class switching, somatic mutation, and probably production of plasma-cell precursors (1–4). In contrast to the TD patterns of B-cell activation, which are associated with GC development, some T-cell-independent (TI) antigens, such as the TI type I (TI-I) bacterial lipopolysaccharide, and the TI-II type antigens, polyvinylpyrrolidone and dinitrophenol-Ficoll, are reported to induce only minimal or no GC development (5). It is, therefore, generally believed that B cells respond to TI antigens solely or predominantly by the plasma cellular reaction, generating plasmablasts and plasma cells located at the periphery of the periarteriolar lymphoid sheaths (PALS).

Native dextran N279 is a near-linear macromolecule of glucose with 90% α(1→6), 5% α(1→), and 5% α(1→3) linkages (6), derived from *Leuconostoc mesenteroides* NRRL B512. With the precisely defined site-filling epitope of [Glc(α1→6)]$_n$, α(1→6)dextrans (DEXs) have served as a classical system in mapping antibody-combining sites and probing the repertoire of antibodies to a single antigenic determinant (7, 8). The structural simplicity of this carbohydrate antigen also provides a model system for studying complex immune responses to bacterial infection.

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In mice, DEX induces a classical TI (type II) pattern of antibody response, with IgM and IgA isotypes predominating and a few IgG3 (9–11). Nude mice can generate anti-DEXs (11), whereas *Xid* strains give no responses to this antigen (12, 13). Unlike protein antigens, native DEX induces no significant class switching to IgG isotypes upon the second stimulation. However, antibody responses to DEXs may persist for long periods after a single injection (10, 14).

Extensive studies of anti-DEX hybridomas and myelomas have revealed a broad repertoire of antibodies using multiple heavy chain variable region ($V_H$) and κ chain variable region ($V_\kappa$) genes (8). Surprisingly, a number of the DEX-specific monoclonal antibodies (mAbs) show multiple point mutations and higher affinities (15), a pattern normally associated with hypermutation and TD responses. In this report, we demonstrate that, in striking contrast to previous studies of TI antigens, DEX can induce the formation of GCs. Interestingly, we do not find a similar induction of GCs in the response to the related α(1→3)α(1→6)dextran, B1355S. Thus some, but not all, TI antigens can induce GCs. The involvement of GCs in the DEX response may account for some of the extensive V region diversity characteristic of this response.

## MATERIALS AND METHODS

**Mice.** BALB/c mice 3–8 months of age were from the National Institutes of Health or bred and maintained in the animal facilities at Columbia University.

**Antibodies and Reagents.** Fluorescein isothiocyanate (FITC)-conjugated DEX ($^{Fl}$DEX), and alkaline phosphatase-conjugated anti-mouse IgM, anti-mouse IgA, and anti-mouse IgG Fc were purchased from Sigma. Biotin-conjugated (bi) dextrans N279 and B1355S were prepared using biotin-LC-hydrazide (Pierce). HPLC-purified Fab fragment of anti-DEX mAb 3.4.1G6 (11) and intact 16.4.12E (16) were conjugated using biotin-*N*-hydroxysuccinimide ester (BRL) as described (17) Phycoerythrin-conjugated anti-CD3 and anti-GR-1 (8C5) were obtained from PharMingen. Allophycocyanin-conjugated anti-B220 (RA3-6B2) was purified and prepared as described (17). FITC-conjugated peanut agglutinin ($^{Fl}$PNA) was obtained from Vector Laboratories. Avidin was conjugated with Texas red (TR; Molecular Probes), as described (17).

**Antigens and Immunization.** Animals were immunized i.v. with 10 μg of native DEX N279 or 10 μg of α(1→3)α(1→6)-dextran B1355S.

**Cell Staining and Fluorescence-Activated Cell Sorter (FACS) Analysis.** Preparation of single-cell suspensions, four-color staining, and FACS data analysis have been described

Abbreviations: ASC, antibody-secreting cell; bi, biotin conjugated; DEX, α(1→6)dextran; FACS, fluorescence-activated cell sorter; Fl, fluorescein conjugated; GC, germinal center; mAb, monoclonal antibody; PALS, periarteriolar lymphoid sheath; PNA, peanut agglutinin; TD, T-cell dependent; TI, T-cell independent; TR, Texas red; V region, variable region; H, heavy chain.

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in detail (18). Analyses were carried out on a dual laser FACStar$^{PLUS}$ modified with a fifth fluorescence parameter. All experiments were done using propidium iodide at 1 μg/ml to identify dead cells, which were excluded in the third fluorescence channel (FL 3-1) from the first (488 nm) laser.

**ELISA-Spot Assays.** Isotype-specific ELISA-spot assays were used to quantify the number of DEX antibody-secreting cells (ASCs). The assays were carried out as described (19) with the following modifications: 96-well microtiter plates were coated with dextran N279 at 1 μg/ml. Bound anti-DEX antibodies were revealed with alkaline phosphatase-conjugated antibodies specific for IgM, IgG, or IgA.

**Preparation of Spleen Sections and *in Situ* Immunofluorescence.** Spleens were embedded in Tissue-Tek OCT compound (Miles) and frozen in dry-ice-cooled 2-methylbutane (Aldrich). Cryostat sections (7 μm) were cut and mounted on gelatin-coated slides, air-dried for 30 min, fixed in ice-cold acetone for 15 min, air-dried again for 30 min, and then stored at −20°C until use. All staining was done in a humidified chamber shielded from light. After blocking with phosphate-buffered saline (PBS)/3% (wt/vol) bovine serum albumin, sections were incubated for 30 min with biotinylated or fluoresceinated reagents as indicated and then incubated with avidin-conjugated TR for 15 min with washings after each staining. The sections were rinsed in distilled water and mounted with Gel/Mount (Biomedia, Foster City, CA). A dual-band filter (Omega Optical, Brattleboro, VT) for simultaneous viewing of fluorescein and TR was used for analysis.

## RESULTS

**GC Formation in Primary Responses to DEX.** We used a $^{Fl}$DEX to identify and localize DEX-specific B cells in spleen sections by immunohistology. Similarly, B cells in GCs were identified by their staining with $^{Fl}$PNA or $^{bi}$PNA (20). Examples of spleen sections costained with $^{Fl}$DEX and $^{bi}$PNA (revealed by TR-avidin) are illustrated in Fig. 1 *A–D*. In unimmunized animals, we have never observed DEX staining in GCs. Fig. 1*A* shows a secondary follicle of an unimmunized mouse revealed by the $^{bi}$PNA (red) staining; however, there is no $^{Fl}$DEX-positive staining inside or outside of the follicle. At day 5 after immunization, clusters of $^{Fl}$DEX-positive cells (green) appear in the PALS area outside a follicle (Fig. 1*B*). The intensive positive cytoplasmic staining suggests that these cells are DEX-specific ASCs. In addition, a low level of $^{Fl}$DEX staining is seen within a developing GC. By day 11 (Fig. 1 *C* and *D*), both PNA$^+$ (red) cells and double-positive cells staining with both $^{bi}$PNA and $^{Fl}$DEX (greenish-yellow) are seen in well-developed GCs. The former could be dividing centroblasts, which are surface immunoglobulin-negative, whereas the latter may be surface immunoglobulin-positive centrocytes derived from them (2). A cluster of DEX$^+$ ASCs (green) is again seen between the two GCs in Fig. 1*D*.

The results of the immunohistology were confirmed by FACS analyses of spleen cells from BALB/c mice at day 0 (unimmunized), day 4, and day 12 to day 14 after injection. In the spleen, the number of cells responding to any single antigenic determinant represents an extremely small percentage (0.01–0.5%) of the B cells. Following a strategy of multiparameter FACS analysis applied in an analysis of phosphocholine-specific B cells (S.M.W., A. B. Kantor, and A.M.S., unpublished data), we have utilized $^{Fl}$DEX to identify DEX-specific (DEX$^+$) B cells in the spleen. In this procedure, dead cells and non-B cells, such as T cells and granulocytes, stained with propidium iodide and anti-CD3 and anti-Gr-1 mAbs, respectively, were gated out during data analyses. By using this approach, which also removes cells that nonspecifically bind antibodies, the lower limit of our detection is ≈0.01% of the cells analyzed.

Although we can specifically detect a population of DEX$^+$ PNA$^-$ B cells in all BALB/c mice, DEX$^+$ PNA$^+$ double-positive cells are only seen 1 week after immunization, similar to the histological analyses. In the nonimmunized animals, only 0.007 ± 0.003% of the cells are DEX$^+$ PNA$^+$, equivalent to background (Fig. 2*B*). By day 4, these cells had only increased to 0.015 ± 0.003% (Fig. 2*C*). However, a distinct population of DEX$^+$ PNA$^+$ cells (0.07 ± 0.056%) is clearly seen in seven BALB/c mice analyzed between day 13 and day 15 after immunization (see day 14, Fig. 2*D*). Thus the appearance of DEX$^+$ PNA$^+$ B cells in the spleen as identified by FACS analysis correlates with the appearance of DEX$^+$ PNA$^+$ cells identified in the GC by immunohistology.

**Deposition of DEX and Its Immune Complexes Occurred Early in the Response Before the Development of GCs.** Localization of DEX was traced by the binding of fluorochrome-labeled anti-DEX mAbs. As early as 2 days after immunization, anti-dextran-specific staining was detected outside of the splenic follicles (data not shown). By day 8, there was an intense anti-DEX (red) staining within the GCs that showed a dendritic staining pattern (Fig. 1 *E* and *F*). In addition, serial sections stained with $^{Fl}$DEX revealed similar staining patterns inside of splenic follicles, consistent with development of DEX–anti-DEX immune complexes associated with the follicles after immunization.

Anti-DEX (red)-positive cells were also seen in the splenic marginal zone, forming a ring around the follicle (Fig. 1*E*). However, in contrast to the DEX–anti-DEX double-positive staining observed inside of splenic follicles, neither $^{Fl}$DEX nor $^{bi}$DEX revealed a similar pattern of marginal zone staining (Fig. 1 *B–D*), indicating that these DEX molecules were not associated with immune complexes. This is similar to the finding by Humphrey (21) who showed that injected $^{125}$I-labeled DEX was captured by splenic marginal zone macrophages (21).

**Time Course of Splenic ASCs in Response to Native Dextran N279.** The ASC response (as measured by ELISA-spot assay) to a single injection of DEX is long lasting (Fig. 3). In unimmunized animals, there is a background level of 6 ASCs per $10^6$ cells. After immunization, the response peaks at day 4 (390 ASCs per $10^6$ cells) and then rapidly decreases to 25% of the peak. This reduced response (≈100 ASCs per $10^6$ cells) persists out to day 28 (last time tested). As expected the ASC response is almost exclusively of the IgM and IgA isotypes with <2% IgG (Fig. 3).

The persistence of the ASCs is also seen in the analyses of the splenic sections. By immunohistology, the ASCs that are localized in the PALS are, as expected, PNA$^-$ and show a characteristic intense cytoplasmic staining with $^{Fl}$DEX (Fig. 1 *B* and *C*). These ASCs are first detected by day 2, peak at day 4 to day 5 (Fig. 1*B*), and are still present 28 days after immunization (last time tested). FACS sorting of DEX$^+$ PNA$^+$ and DEX$^+$ PNA$^-$ populations of B cells showed that, as expected, only the latter population contains DEX-specific ASCs, as measured in the ELISA-spot assay (data not shown).

**Dextran B1355S Induces Predominantly Plasma-Cell Responses in Spleen.** To see whether a structurally related bacterial polysaccharide may induce a similar pattern of splenic B-cell responses, the primary immune response to dextran B1355S [referred to as α(1→3)α(1→6)dextran] was also analyzed. Although dextrans B1355S and N279 are produced by strains of *Leuconostoc mesenteroides*, they differ in their composition of glycosidic linkages. B1355S is the prototype of dextrans having significant proportions of non-α(1→6) linkages, with α(1→3)α(1→6)-linked glucoses as a predominant antigenic determinant (6). The two dextrans elicit distinct repertoires of antibodies that do not cross react and differ in $V_H$ usage and light-chain isotype (8, 22, 23).

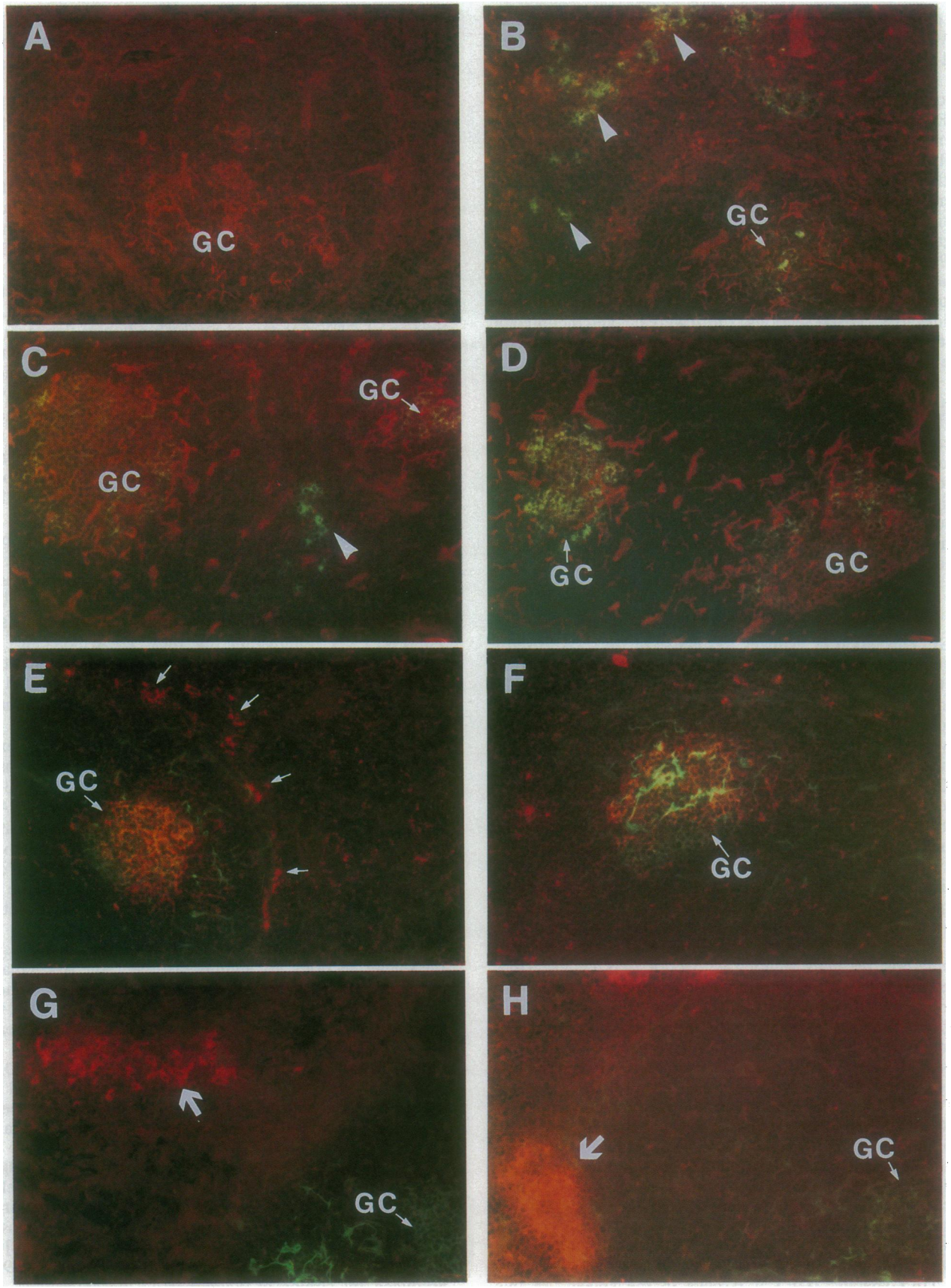


FIG. 1. Antigen-specific GCs are induced after immunization with DEX. (*A–D*) Frozen sections from BALB/c spleens at various times after DEX immunization were stained with $^{Fl}$DEX and $^{bi}$PNA revealed with TR-avidin. (*A*) Unimmunized mouse on day 0. (*B*) Day 5 after immunization. (*C* and *D*) Day 11 after immunization. Arrowheads indicate DEX$^+$ ASCs in the PALS. (*E* and *F*) Frozen sections from BALB/c spleens at various times after DEX immunization were stained with $^{bi}$Fab-3.4.1G6 ($^{bi}$anti-DEX; red) and $^{Fl}$PNA (green). (*E*) Day 8 after immunization; arrows indicate anti-DEX$^+$ staining in the marginal zone. (*F*) Day 11 after immunization. (*G* and *H*) Frozen sections from BALB/c spleens day 7 after immunization with B1355S $\alpha(1\rightarrow3)\alpha(1\rightarrow6)$dextran were stained with $^{Fl}$PNA and $^{bi}$B1355S [$\alpha(1\rightarrow3)\alpha(1\rightarrow6)$dextran].

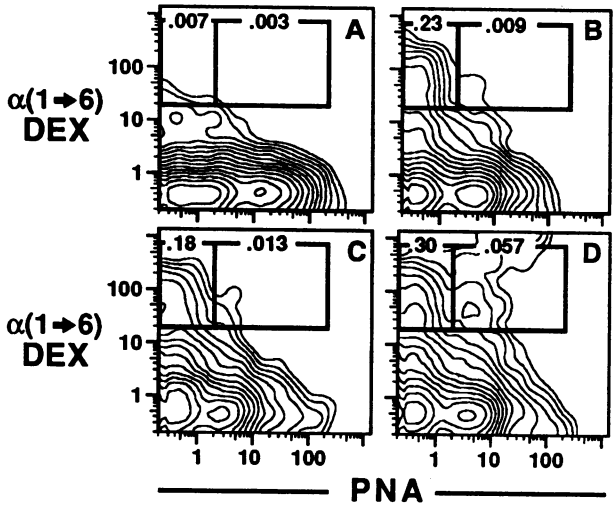


FIG. 2. Distinct population of DEX$^+$ PNA$^+$ cells appears ≈2 weeks after immunization. BALB/c spleen cells isolated from unimmunized mice (*A* and *B*) or mice 4 days (*C*) or 14 days (*D*) after immunization with DEX were stained with $^{Fl}$DEX (except *A*), $^{bi}$PNA, and allophycocyanin-conjugated anti-B220. In addition, cells were stained with phycoerythrin-conjugated anti-CD3 and phycoerythrin-conjugated anti-Gr-1, which were used to exclude T cells and granulocytes from the analysis. (*A*) Background control in which $^{Fl}$DEX was excluded from the staining mixture. (*B–D*) The DEX$^+$ cells are all B cells, as determined by their positive staining for B220 (data not shown). The data are presented as 50% logarithmic plots of CD3$^-$ Gr-1$^-$ cells. To ensure statistical significance, data on 200,000 splenocytes (which includes 100–300 DEX$^+$ PNA$^+$ cells in *D*) were collected for each sample.

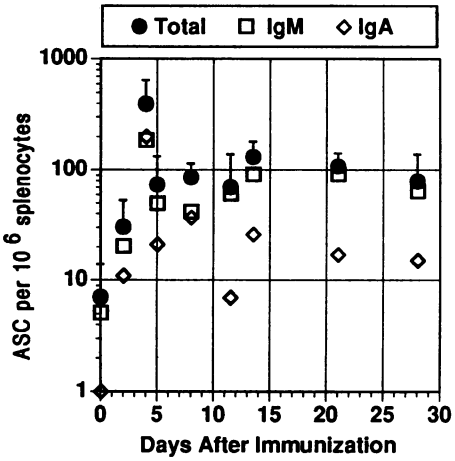


FIG. 3. Persistence of DEX-specific ASCs in spleen after a single i.v. injection of native dextran N279. BALB/c mice were immunized i.v. with 10 μg of DEX and at the indicated times spleen cells were isolated and the number of IgM (□), IgA (◇), and IgG (not shown) DEX-specific ASCs was determined by isotype-specific ELISA-spot assays. Error bars are given for the total (●, IgM+IgA+IgG) ASCs. The percentage of IgG ASCs never exceeded 2% of total at any point tested. For each time point, two to nine animals were tested.

BALB/c mice were immunized by a single intravenous injection of 10 μg of α(1→3)α(1→6)dextran. By day 7, a typical plasma-cell response was induced, characterized by formation of large clusters of B1355S$^+$ cells or foci in the PALS outside of the GCs (Fig. 1 *G* and *H*). Staining with various anti-light chain mAbs reveals that these foci are, as expected, predominantly associated with the λ1 light chain (data not shown). In striking contrast to animals immunized with N279 DEX, there were no α(1→3)α(1→6)dextran-specific GCs observed up to day 14. Thus, while both antigens induce a vigorous ASC response, only DEX leads to the induction of GCs.

## DISCUSSION

Antigens have generally been classified as TI based upon their ability to elicit an immune response in athymic (*nu/nu*) mice and more recently on the inability to elicit responses in mice carrying the *Xid* gene defect (24). TD responses typically involve a variety of processes, including hypermutation (affinity maturation), isotype class switching, and GC formation, which are thought to require antigen-specific T-cell help. In this report we have documented that, contrary to the prevailing belief, a classical TI-II polysaccharide antigen can induce the development of GCs. This unexpected finding fundamentally alters our concepts of which factors are important in the generation of GCs and raises questions as to the relative importance of GCs and antigen-specific T-cell help in the process of hypermutation and class switching.

Early studies by Thorbecke and coworkers (19) indicated that in *nu/nu* mice, development of GCs was dependent upon the adoptive transfer of thymocytes. Our data do not preclude the involvement of any T cells in the development of the anti-DEX GCs. Although the data indicate that classical antigen-specific T-cell help central to TD responses may not be required, it is possible that other (nonspecific or idiotype-specific) T cells are necessary for the development of the GCs in this response. The unique GC response to DEX will allow us to dissect which T cells may be involved in this process.

The antibodies produced in response to dextran N279, a structurally defined polysaccharide, with α(1→6)-linked internal linear chains predominating, have been extensively characterized (7, 8). By the criteria defined above, N279 DEX is a classic TI type II antigen. However, we show that a single i.v. injection induces well-developed distinct GCs that can be visualized in splenic tissue sections by staining with fluorochrome conjugates of PNA and DEX. In addition, PNA$^+$ DEX$^+$ B cells could also be identified by FACS in the spleens of animals after immunization with DEX.

In striking contrast to N279 (DEX), which induces both an ASC response and a GC response, B1355S [α(1→3)α(1→6)-dextran] induces a vigorous ASC response with no evidence of GC formation. The distinct immune responses to the two polysaccharides, differing with respect to antibody repertoire and their pathways of B-cell activation, demonstrate that not all TI antigens can induce GC and suggests that, among other factors, differences in the responding B cells may play a role in determining whether GCs are induced. Förster and Rajewsky (25) demonstrated that in BALB/c mice the dominant λ1 response to B1355S α(1→3)α(1→6)dextran is derived, at least in part, from cells of the B-1 lineage. It is possible that the lineage origin (B-1 or conventional) of the B cells responding to a given antigen may influence the generation of GCs.

While the molecular mechanisms of somatic hypermutation are still poorly understood, an association between GC reaction and hypermutation has been well documented (4). However, these studies have all involved TD antigen responses. In the DEX response, it is now possible to address the relationship of hypermutation, GC reaction, and/or T-cell help. In our previous studies, we found (15, 26–28) that, in the primary BALB/c anti-DEX response, point mutations occur at high frequency in the V regions of the major group of DEX mAbs (the $V_H$19.1.2 family) resulting in high-affinity dextran binding (15, 26), loss of idiotypic determinants (27), and generation of N-glycosylation sites in $V_H$-CDR2 (28). Although unexplained at the time, given the TI nature of the antigen, these data suggested that hypermutation had occurred in diversifying the V regions of $V_H$19.1.2 mAbs. Since

DEX can induce GCs, it is possible that hypermutation can occur in the absence of classical antigen-specific T-cell help, requiring only the formation of GCs, which among other factors, provide the appropriate environment for selection by antigen. Analysis of other TI antigenic responses will be required to test this hypothesis.

Although the response to DEX has some of the characteristics of a TD response (induction of GCs and possibly hypermutation), the isotype profile of the antibodies produced is typical of TI-II antigens—predominantly IgM and IgA with little IgG. The induction of DEX-specific GCs without switching to IgG isotypes indicates that although isotype class switching may occur in GCs, the presence of GCs is in itself not sufficient to induce IgG class switching. This is not surprising considering the well-documented requirement for T-helper-cell-derived cytokines in the class switch to IgG (29).

Retention of DEX and its immune complexes in follicles may be crucial for the development of dextran-specific GCs. Dendritic cells in follicles, which retain antigen–antibody complexes by their Fc and C3 receptors, are believed to be important to the generation of GCs (30). It has recently been demonstrated that GC cells are programmed to die by apoptosis unless they receive a positive signal for rescue. This signal *in vivo* is probably generated by interaction with antigen held as immune complexes on follicular dendritic cells. Native dextran N279 is a large molecule with multiple repeats of $\alpha(1\rightarrow6)$-linked glucose and is noncatabolizable in mammals (6). Therefore, dextran immune complexes can be readily generated that persist in the follicles much longer than rapidly catabolized protein antigens. The persistence of these DEX–anti-DEX immune complexes may be responsible for both the extended life of the DEX GCs and the ASC response as measured by ELISA-spot assays (Fig. 3). Retention of the dextran molecule by marginal zone macrophages could be also important. Further studies are required to reveal the relative contribution of these events to the prolonged anti-DEX response.

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