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Immunoglobulin G Subclass-Specific Antileishmanial Antibody Responses in Indian Kala-Azar and Post-Kala-Azar Dermal Leishmaniasis

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Antileishmanial antibody responses in the sera of Indian kala-azar (KA) and post-KA dermal leishmaniasis (PKADL) patients were analyzed by enzyme-linked immunosorbent assay (ELISA) and immunoblot experiments using immunoglobulin G (IgG) class- and subclass-specific reagents. All sera showed antileishmanial reactivities in IgG ELISA which followed the order IgG1 > IgG2 > IgG3, with very little IgG4. Immunoblot analysis with IgG class-specific reagents revealed variable patterns of reactivity by KA and PKADL sera, although certain common bands around the 60- to 63-kDa and 28-kDa regions were discernible. Sera from antimony-unresponsive KA cases, on the other hand, strongly recognized two bands at around 20 to 22 kDa, in addition to other bands in the high-molecular-mass region. Further analysis showed that the 28-kDa band was preferentially recognized by the IgG2 isotype, while 20- to 22-kDa and 60- to 63-kDa bands were recognized by the IgG1 isotype. Antibodies belonging to the IgG3 isotype reacted to antigens primarily in the region of 14 to 34 kDa and persisted in patients even several months after cure. Immunoblot studies also revealed the presence of a nonspecific band which arose as a result of binding between a 66-kDa leishmanial antigen and streptavidin. Finally, the results presented in this study suggest that certain leishmanial antigens preferentially stimulate the synthesis of a particular IgG subclass(es), depending on the nature of such antigens or their epitopes.

Visceral leishmaniasis, or kala-azar (KA), is caused by the protozoan parasite *Leishmania donovani* in the Indian subcontinent. The disease is marked by *Leishmania* antigen-specific suppression in the cell-mediated immune component of the host (5, 7, 14, 18, 30, 33). KA patients who have recovered regain their normal cell-mediated immune functions (6, 14, 33) and usually become immune to a subsequent attack of the viscerotropic form of the disease (26). About 20% of the KA patients in India, however, develop post-KA dermal leishmaniasis (PKADL), a dermal recrudescence of the disease, 6 months to several years after an apparent cure of KA (3, 35).

Antileishmanial antibodies belonging primarily to the immunoglobulin G (IgG) class have already been demonstrated in both KA (12) and PKADL (15) sera. However, their relevance in the prediction of the course of leishmanial infection is yet to be established. Recent studies (21–24) suggest that the course of infection is probably determined by an interplay of different cytokines, such as interleukin-2, interleukin-4, and gamma interferon, etc., produced by *Leishmania*-reactive subpopulations of T cells. Some of these cytokines are also involved in the switching mechanism between different isotypes of immunoglobulin heavy chains (25, 28). Little information is currently available about the isotype-specific antileishmanial antibody responses in KA and PKADL. In the present study, we have analyzed sera from patients with acute KA and PKADL to determine the differences, if any, in the IgG subclass distribution patterns of their antileishmanial responses. Further, the identification of leishmanial antigens as markers for such subclass-specific humoral immune responses has been carried out by immunoblotting experiments.

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MATERIALS AND METHODS

Collection of sera. Forty KA patients admitted to the Calcutta School of Tropical Medicine and the Canning Rural Hospital, West Bengal, India, were included in this study. All of these cases were diagnosed as KA after careful clinical and laboratory investigations, and the diagnosis was confirmed by the demonstration of amastigotes (*L. donovani* bodies) in bone marrow smears. The majority of these patients were found to be responsive to treatment with stibanate (sodium antimony gluconate), but four patients did not respond to stibanate therapy and had to be treated subsequently with the drug pentamidine. Blood samples were collected from the KA patients before chemotherapy (with either stibanate or pentamidine). Convalescent-phase sera were collected from a few of these KA patients after successful chemotherapy. Blood samples were also obtained from 16 patients suffering from PKADL as evidenced by the demonstration of amastigotes (*L. donovani* bodies) in skin biopsy material. Blood samples from healthy individuals living in areas where KA is not endemic were collected and used as controls. Sera obtained from all blood samples thus collected were stored at −20°C until used.

Preparation of leishmanial antigens. *Leishmania donovani* ASI promastigotes were cultivated in modified Ray's medium (29) supplemented with brain heart infusion (Difco, Detroit, Mich.) and rabbit blood for the large-scale preparation of parasites. Promastigotes grown on the surface of the solid medium as slimy layers were harvested by gentle washing with normal saline. The harvested parasite suspension was centrifuged at 500 × *g* for 15 min, and the supernatant was discarded. The parasite pellet was resuspended again in normal saline and centrifuged, and the washing procedure was repeated twice. Finally, parasites were resuspended (10^9 parasites per ml) in 10 mM Tris buffer (pH 7.4) containing 20 mM NaCl, 10 mM EDTA disodium salt (Glaxo, Bombay, India), 2 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, Mo.), 2 mM iodoacetamide (Sigma), and 50 μM leupeptin (Sigma). Leishmanial crude soluble antigen (CSA) was prepared essentially by the methodology of Ghose et al. (12). For this, about 5 ml of the parasite suspension was homogenized with a Dounce homogenizer (Wheaton, Millville, N.J.) and subsequently sonicated at 4°C by using an Ultrasonicator (Braunsonic 1510; B. Braun AG). The homogenized material was centrifuged at 6,000 × *g* for 15 min at 4°C, and the clear supernatant was collected and used as the CSA. The protein content of the sample was estimated by the modified Lowry method (27).

Leishmania promastigotes were also grown in the liquid culture medium F-12

TABLE 1. Antileishmanial antibody titers of the IgG class in sera of KA and PKADL patients as determined by ELISA

Group	No. studied	No. with IgG ELISA titers of[a]:						
		800	1,600	3,200	6,400	12,800	25,600	≥51,200
KA	40	4	2	4	0	4	4	22
PKADL	16	2	1	3	3	6	1	0

[a] Antibody titers were determined against leishmanial CSA. IgG ELISA titers in sera obtained from 15 normal healthy subjects were ≤200.

(Sigma) supplemented with 5% (vol/vol) fetal bovine serum (Inovar, Gaithersburg, Md.), 10% (vol/vol) normal rabbit blood cell lysate, 1.18 g of $NaHCO_3$ per liter, and 50 μg of gentamicin (Hi-media, Bombay, India) per ml (pH 7.2 to 7.4). Freshly harvested promastigotes were used as the antigen in immunoblotting experiments.

Enzyme-linked immunosorbent assay (ELISA). The micro-ELISA method was used for the determination of antileishmanial antibody titers in KA and PKADL sera (12). For this, wells of micro-ELISA Maxisorp plates (Nunc, Roskilder, Denmark) were sensitized by overnight incubation at 4°C with 5 μg (each well) of CSA protein in 100 μl of coating buffer. The plate was subsequently washed with phosphate-buffered saline (PBS) (pH 7.4) containing 0.05% (vol/vol) Tween 20 (PBS-Tween), and 200 μl of a 3% (wt/vol) solution of bovine serum albumin (BSA) (Sigma) in PBS was added to each well to saturate all unbound sites. Following incubation and washing, 100-μl amounts of test serum, serially twofold diluted with 0.5% (wt/vol) BSA in PBS starting from an initial dilution of 1:200, were added, and the plate was incubated for 1 h at room temperature. Next, the plate was washed and 100 μl of goat anti-human IgG-peroxidase conjugate (Sigma), diluted to 1:5,000 with 0.5% (wt/vol) BSA in PBS, was added to each well. Following washing, 100 μl of a substrate solution (*o*-phenylenediamine [1 mg/ml] in 0.1 M citrate buffer [pH 4.5] and 5 μl of 30% [vol/vol] H_2O_2 [1 μl/2 ml]) was added. After 15 to 20 min, 100 μl of H_2SO_4 was added to each well, and the A_{492} was measured in a micro-ELISA reader (Anthos 2001; Anthos Labtec Instruments, Salzburg, Austria). Titers were expressed as the highest dilution of the serum that showed definite color development (an A_{492} of ≥0.2) in the assay (34).

IgG subclass-specific antileishmanial antibody responses were also measured in microtitration plates with CSA as the antigen. The procedure followed was very similar to that described above except that instead of the peroxidase-antibody conjugate, 100 μl of appropriately diluted biotin-conjugated mouse monoclonal antibodies to human IgG1, IgG2, IgG3, and IgG4 (Sigma) were added to each well for isotype-specific antibody measurements. Following incubation and washing with PBS-Tween, 100 μl of streptavidin-peroxidase (Sigma), diluted to 1:10,000 with PBS–0.5% BSA, was added; this was followed by the addition of 100 μl of the substrate solution. The results were recorded as described above.

Immunoblotting. The reactivity of the IgG class of antileishmanial antibodies to promastigote antigens was studied by immunoblot experiments (37). For this, *L. donovani* whole promastigotes (10^7 per lane) were dissolved in sample buffer consisting of 10 mM Tris-HCl (pH 6.8) containing 2% (wt/vol) sodium dodecyl sulfate (SDS) (Sigma), 5% (vol/vol) 2-mercaptoethanol (Sigma), 10% (wt/vol) sucrose (SRL, Bombay, India), and 0.002% bromophenol blue (SRL). The mixture was boiled for 10 to 15 min in a water bath, cooled to room temperature, and subjected to electrophoresis with a separating gel (10 by 13.8 cm) of 12.5% acrylamide. Electrophoretic transfer of the separated antigens from the SDS-polyacrylamide gel to a nitrocellulose membrane (0.2-μm pore size; Sigma) was carried out with a constant current of 170 mA. The blotted strips were treated with a 3% (wt/vol) BSA solution in PBS and incubated with appropriate dilutions of test serum for 1.5 h at room temperature. Next, the strips were washed, treated with goat anti-human IgG-peroxidase conjugate (Sigma), and developed with the substrate solution 3,3′-diaminobenzidine (Sigma) in Tris-NaCl buffer (pH 7.4) containing 30% H_2O_2 (1 μl/2 ml).

Immunoblot analysis of leishmanial antigens was also carried out by using patient sera and reagents specific for different subclasses of human IgG. The methodology was essentially similar to that described above except that following incubation with patient sera, the strips were treated with biotinylated mouse monoclonal antibodies to human IgG1, IgG2, IgG3, and IgG4. Next, the strips were developed by successive treatments with streptavidin-peroxidase conjugate (Sigma) and the substrate solution 3,3′-diaminobenzidine as described above.

RESULTS

ELISA reactivities of IgG class- and subclass-specific antileishmanial antibodies in sera of KA and PKADL patients. Sera obtained from 40 KA patients and 16 PKADL patients were initially screened for antileishmanial antibodies by ELISA with IgG class-specific conjugates (Table 1). Elevated antileishmanial antibody titers were demonstrable in sera from all of these active cases, although the antibody titers were higher in the majority of KA sera than in sera from PKADL patients.

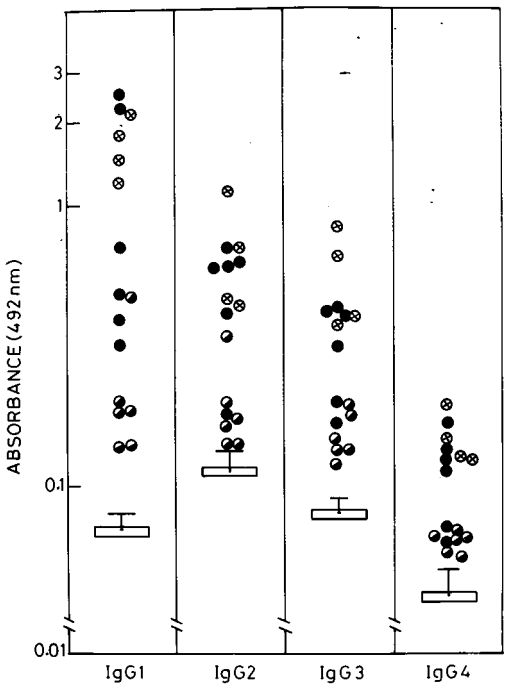

FIG. 1. IgG subclass ELISA reactivities of sera from KA patients responsive (●) and unresponsive (⊗) to treatment with antimony. Reactivities of sera from PKADL patients are also shown (○). Absorbance values were obtained at an antiserum dilution of 1:400. Mean absorbance values for control sera and their standard deviations are shown as boxes and vertical lines, respectively.

The IgG isotype distributions of antileishmanial antibodies in 10 KA patients (including four cases that were unresponsive to stibanate therapy) and 6 PKADL patients were further studied by ELISA with IgG subclass-specific conjugates. The results obtained at an antiserum dilution of 1:400 are presented in Fig. 1. Antileishmanial antibodies belonging to all four subclasses were demonstrable in the sera of KA patients. This was true for both stibanate-responsive and -unresponsive patients. Similar increases in antibody levels, although lesser in magnitude than those in KA sera, were also noted in PKADL sera. The antileishmanial antibody levels followed the order IgG1 > IgG2 > IgG3 > IgG4, with the differences between the successive subclasses of IgG being statistically significant ($P < 0.05$). In fact, the IgG4 antibodies were only marginally detectable in some KA and PKADL sera. The analysis of data based on the determination of antileishmanial antibody titers of different IgG subclass also provided similar information. Representative results obtained with one stibanate-unresponsive KA patient, one responsive KA patient, and one responsive PKADL patient are shown in Fig. 2.

Immunoblot reactivities of IgG class- and subclass-specific antileishmanial antibodies in sera of KA and PKADL patients. The immunoblot reactivities of sera obtained from six KA patients and one PKADL patient to leishmanial whole-cell antigen was studied by using IgG-specific conjugates (Fig. 3). It is evident that sera obtained from different patients showed variable patterns of reactivity, although the majority of these sera recognized certain common bands around the 60- to 63-kDa and 28-kDa regions. Sera obtained from stibanate-unre-

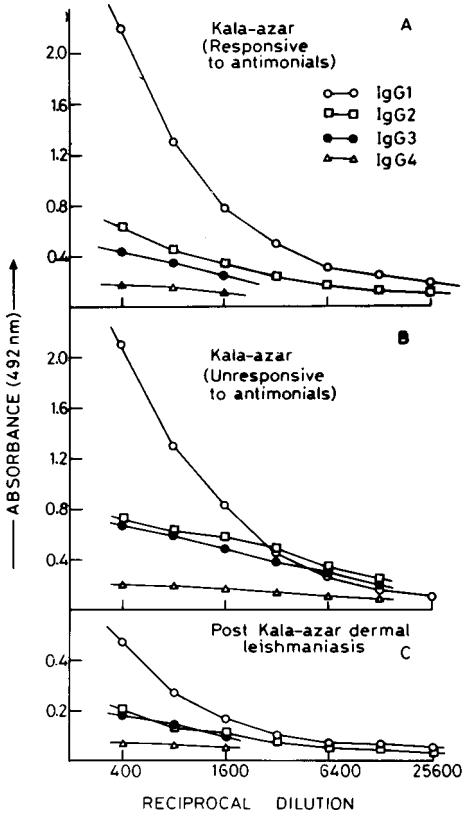

FIG. 2. IgG subclass-specific antileishmanial ELISA titers of representative sera from KA and PKADL patients.

sponsive patients, on the other hand, strongly recognized two bands at around 20 to 22 kDa, in addition to other bands in the high-molecular-mass region (Fig. 3, lane 5; Fig. 4, lanes 1 to 3). Normal human serum, however, failed to show any reactivity (Fig. 4, lane 4), thereby suggesting specific recognition of leishmanial antigens by KA sera in these immunoblot experiments.

Further analysis of *L. donovani* promastigote antigens rec-

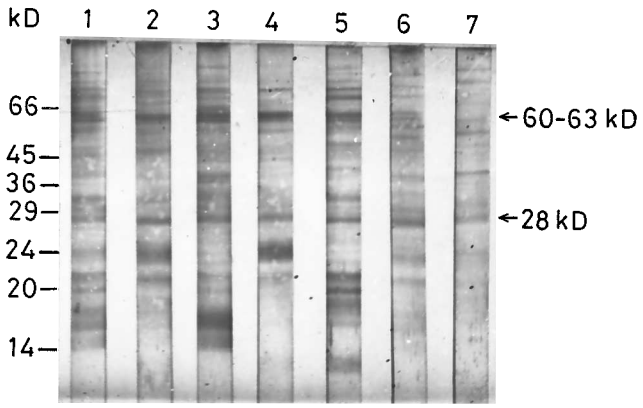

FIG. 3. Immunoblot reactivities of different KA (lanes 1 to 6) and PKADL (lane 7) sera against *L. donovani* promastigote antigens. IgG class-specific conjugate was used. All patients except one (lane 5) were responsive to antimony treatment. Molecular mass markers used were BSA (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), and lactalbumin (14 kDa).

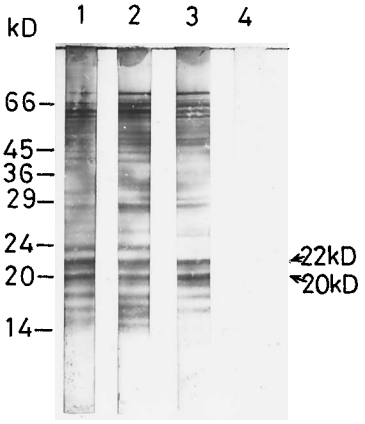

FIG. 4. Immunoblot reactivities of sera from three KA patients (lanes 1 to 3) unresponsive to antimony. Lane 4 represents the reactivity of a control normal serum. IgG class-specific conjugate was used. Molecular mass markers are as described in the legend to Fig. 3.

ognizable by different IgG subclass antibodies in KA sera was carried out by immunoblot experiments. Figure 5 shows the immunoblot reactivity patterns of sera obtained from antimony-responsive as well as -unresponsive KA patients. Antibodies belonging to different IgG subclasses showed variable patterns of reactivity to leishmanial antigens, except that they all recognized common bands around the 60- to 66-kDa region. The 66-kDa band, however, was found to be nonspecific, as it was generated by all sera tested, including normal human serum (data not shown). Further analysis revealed that the band was generated primarily as a result of recognition of a 66-kDa leishmanial antigen(s) by streptavidin. The recognition of bands around the 60- to 63-kDa region, however, was specific and mainly associated with the IgG1 subclass of antibodies. As noted above, the antimony-unresponsive serum recognized additional bands at around 20 to 22 kDa by antibodies belonging to the IgG1 subclass. The IgG2 antibodies, on the other hand, strongly recognized the 28-kDa band, which was only poorly

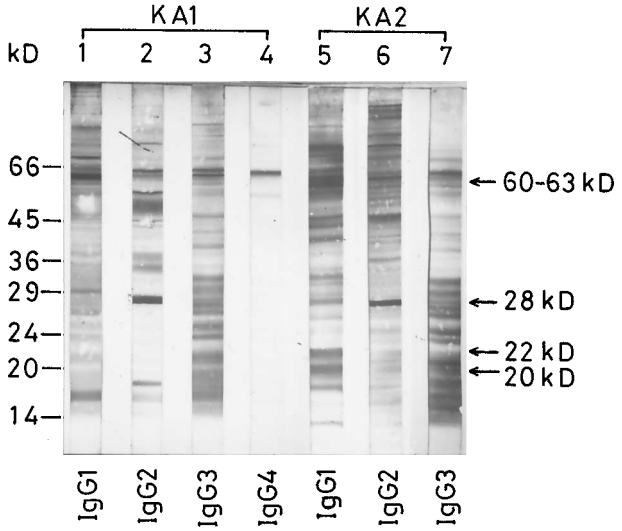

FIG. 5. Immunoblot reactivities of KA sera against promastigote antigens with IgG subclass-specific conjugates. Lanes 1 to 4, antimony-responsive KA serum (KA1); lanes 5 to 7, KA serum (KA2) unresponsive to antimony. Molecular mass markers are as described in the legend to Fig. 3.

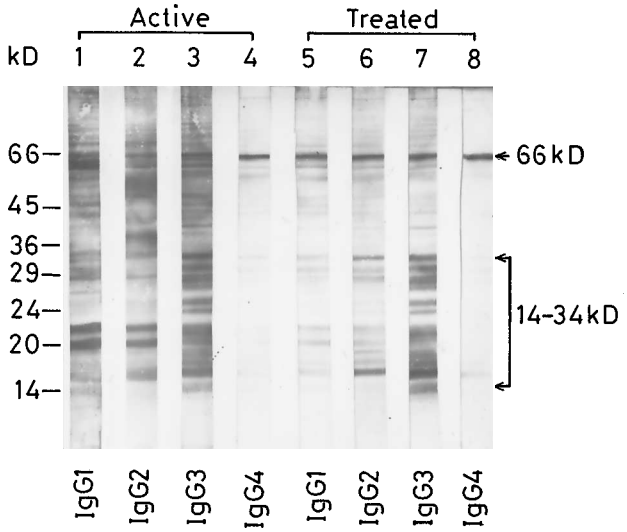

FIG. 6. Immunoblot reactivities of acute- and convalescent (6 months)-phase sera collected from a KA patient. IgG subclass-specific conjugates were used. Lanes 1 to 4, acute-phase KA serum; lanes 5 to 8, convalescent-phase serum. Molecular mass markers are as described in the legend to Fig. 3.

recognized by other IgG subclasses. The IgG3 antibodies showed broad reactivity to leishmanial antigens predominantly in the region between 14 and 34 kDa, while the IgG4 isotype failed to show any significant reactivity to leishmanial antigens, if one takes into consideration the nonspecific nature of the 66-kDa band. Comparable results were also obtained with some other KA sera tested by us (data not shown).

Immunoblot analysis of sera obtained from a KA patient during the active phase of the disease and after successful chemotherapy was carried out with IgG subclass-specific conjugates (Fig. 6). The acute-phase serum contained antibodies of different subclasses which showed considerable reactivity to various leishmanial antigens, particularly at 60 to 63 kDa (IgG1), 20 to 22 kDa (IgG1 and IgG2), and 14 to 34 kDa (IgG3). Serum collected from the same patient even 24 weeks after recovery retained broad reactivity (mostly IgG3) to leishmanial antigens around the 14- to 34-kDa region, while other

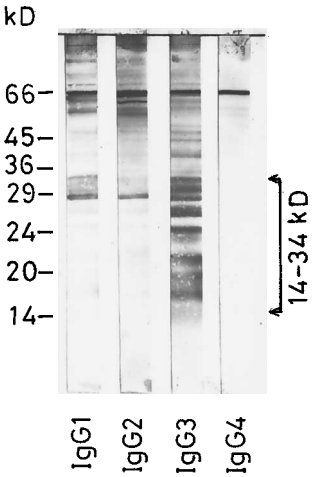

FIG. 7. Immunoblot reactivity of serum from a PKADL patient against promastigote antigens. IgG subclass-specific conjugates were used. Molecular mass markers are as described in the legend to Fig. 3.

subclass-specific reactivities almost disappeared. The subclass-specific immunoblot reactivity (Fig. 7) of serum from a PKADL patient (with a history of KA about 2 years earlier) was found to be somewhat similar to that observed with the serum obtained from the cured KA patient, with broad reactivity being restricted primarily to the IgG3 subclass.

DISCUSSION

Our earlier studies (12, 15) demonstrated that the IgG class is the major class of antibodies present in the sera of Indian KA and PKADL patients. This conclusion was based on the estimation of total IgG content as well as the determination of antileishmanial antibody titers. Variations in the antileishmanial antibody titers (Table 1) in KA sera are likely to be a reflection of the differences in the duration and severity of the illness (11, 12). The PKADL patients, on the other hand, displayed a more restricted serological response, which is consistent with the localized nature of the disease profile (15).

The results presented here extend our earlier observations (12) by showing that antileishmanial antibodies belong primarily to the IgG1, IgG2, and IgG3 subclasses and that their levels follow the order IgG1 > IgG2 > IgG3, with very little IgG4. These results are in partial agreement with the information available so far from studies with Sudanese (9) and Brazilian (41) KA patients, in which elevation of the serum IgG1 and IgG3, but not IgG2, levels was noted. In certain chronic parasitic diseases, the IgG4 subclass response was found to be predominant and was also accompanied by the elevation of serum IgE levels (19, 20). Although elevation of IgE and interleukin-4 in KA sera was already documented (41), the present study, as well as earlier studies (9, 41), shows minimal involvement of IgG4 in active KA.

The IgG1 and IgG3 classes of antibodies are known to possess high levels of complement-fixing and opsonizing activities (4). Thus, the elevation of total (41) as well as *Leishmania*-specific IgG1 and IgG3 antibodies may be responsible for the observed lowering of C3 levels (12), tissue damage, and other inflammatory reactions (40) noted in active KA. IgG2 antibodies, on the other hand, are poorly complement fixing and opsonizing (38) and therefore are less likely to contribute to such abnormal pathology. Earlier studies (9, 41) failed to document any significant rise of IgG2 levels in the sera of Brazilian and Sudanese visceral leishmaniasis patients, although in the present study antileishmanial antibodies of the IgG2 subclass could be demonstrated in a majority of KA sera. It would be of interest to determine whether the synthesis of IgG2 antileishmanial antibodies is also reflected in a significant increase in total IgG2 levels in the sera of Indian KA patients.

Attempts to identify *L. donovani* antigens recognized by visceral leishmaniasis sera in immunoblot experiments showed considerable variability in the patterns of antigen recognition by infected sera from different geographic regions (1, 8, 31, 32). Leishmanial parasites are known to express a 63-kDa surface glycoprotein (gp63) (2) which is a major antigen recognized by sera from patients with different forms of leishmaniasis, including KA (1, 8, 17, 31). The 60- to 63-kDa band(s) in our immunoblot experiments is likely to arise as a result of recognition of this major surface glycoprotein in different stages of deglycosylation (10). The 28-kDa band needs special mention, as it is recognized by sera from a large number of KA cases, including the stibanate-unresponsive ones (Fig. 3 and 4). A 28-kDa cross-reactive antigen identified in the parasite *L. donovani chagasi* was recognized by sera from Brazilian patients infected with *L. donovani chagasi* as well as *Trypanosoma cruzi* and mycobacteria, but not by control sera (31). Although the 28-

kDa antigen reported here was not recognized by control sera, its cross-reactivity pattern with sera from patients with unrelated parasitic and mycobacterial diseases remains to be established.

Qualitative analyses of immunoblot data suggest that certain leishmanial antigens are preferentially recognized by a particular subclass of antibodies. To our knowledge, this is the first study in which attempts were made to analyze leishmanial antigens with respect to their patterns of reactivity to different heavy-chain isotypes of antibody molecules. Thus, the 28-kDa band appears to be preferentially recognized by the IgG2 isotype, while 20- to 22-kDa and 60- to 63-kDa bands are better recognized by IgG1 and IgG2 isotypes. Although there is suggestive evidence (16) to show that the IgG2 response is produced predominantly against carbohydrate antigens while IgG1 and IgG3 are generally stimulated against protein antigens of certain bacteria, such a generalization may not necessarily be true for all microbial antigens. In fact, leishmanial antigens recognized by IgG1 antibodies appear to be different from those recognized by IgG3, with the antigen recognition patterns of the latter isotype being remarkably similar among different KA and PKADL patients studied. Thus, it appears that the isotype switch of Ig heavy chain may depend on the nature of the antigen(s) or its epitopes and is regulated by cytokines released as a result of a complex interaction between the antigen/epitopes and receptors/major histocompatibility complex of various antigen-processing cells (36, 39).

Antileishmanial antibodies were earlier shown to persist in KA patients even after several months of successful chemotherapy and clinical recovery (13, 14). Our results show that the antileishmanial activity of the serum of a cured KA patient was, more or less, restricted to IgG3 antibodies which recognized antigens around the 14- to 34-kDa region. KA patients are known to show T-cell unresponsiveness to leishmanial antigens during the active stage of the disease, although such reactivity is restored in cured KA patients (5, 14, 18, 33). Thus, it would be of considerable interest to determine whether the same leishmanial antigens that are recognized by antibodies of the cured KA patients are also responsible for stimulation of their T lymphocytes. Further studies are needed to identify and characterize such antigens.

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