# Identification of Paramyosin as Schistosome Antigen Recognized by Intradermally Vaccinated Mice

DAVID E. LANAR, EDWARD J. PEARCE, STEPHANIE L. JAMES, ALAN SHER

**Mice immunized intradermally with extracts of *Schistosoma mansoni* in combination with the adjuvant BCG are significantly protected against subsequent infection with living larval forms of the parasite. Remarkably, these vaccinated animals produce antibodies predominantly against a single parasite protein of molecular weight 97 kilodaltons (Sm-97). A complementary DNA that encodes about half of the Sm-97 molecule has now been cloned and sequenced. Analysis of the deduced amino acid sequence reveals a protein containing periodic repeats of hydrophobic amino acids characteristic of an α-helical coiled-coil structure. The deduced amino acid composition of the cloned gene and several properties of the native protein are similar to that of paramyosin, an α-helical protein that forms the core for myosin filaments in invertebrate muscle. Paramyosin was isolated from *Schistosoma mansoni* adult worms and antibodies to Sm-97 were shown to react with this molecule as well as with a known paramyosin from molluscan muscle.**

A MAJOR GOAL OF RESEARCH ON schistosomiasis is the development of a chemically defined vaccine. Several experimental vaccines based on schistosome extracts or affinity-purified antigens have now been described (*1–5*). Our work has focused on the identification of immunogens responsible for protective immunity induced by intradermal injection of larval or adult extracts of *Schistosoma mansoni* in conjunction with the adjuvant BCG (strain Bacille Calmette Guerin of *Mycobacterium bovis*). This method of antigen presentation is known to selectively stimulate strong T lymphocyte–dependent cell-mediated immunity (*1*). Animals immunized in this manner show statistically significant levels of protection when challenged with living *S. mansoni* cercariae (*1, 6, 7*) as well as potent cell-mediated responses to schistosome antigens (*8*). In contrast, the antibody response elicited by such vaccination is highly restricted (*7*). Indeed, immunochemical analyses reveal that antibodies from mice vaccinated with complex parasite extracts plus BCG predominantly recognize a single antigenic component with a molecular size of 97 kD (*9*). That this antigen (Sm-97) may be important in the induction of protective immunity was indicated by studies in which a subfraction of soluble adult schistosome extract (SWAP) containing the Sm-97 molecule selectively immunized mice against challenge infection (*10*) and by recent experiments (*11*) in which significant protection was achieved by vaccination with the affinity-purified antigen. To investigate the structural properties of the Sm-97 antigen responsible for its selective immunogenicity, we have cloned the gene encoding this molecule by expression screening of a complementary DNA-λgt11 library and examined its nucleotide and deduced amino acid sequence.

Previous experiments indicated that polyvalent antibodies produced by immunization with Freund's adjuvant were more effective than murine vaccine antibodies in screening expression libraries (*12*). For this reason, an antiserum was prepared by immunizing a rabbit with Sm-97 purified by absorption of SWAP to antibodies from mice vaccinated intradermally with SWAP plus BCG (*13*). Similarly, a control antiserum was produced by reacting SWAP with antibodies from mice immunized with BCG alone. These rabbit antisera were then tested by their reaction in vitro with translation products of polyadenylated [poly(A)$^+$] messenger RNA (mRNA) prepared from adult schistosomes. As shown in Fig. 1, the antiserum to Sm-97 immunoprecipitated two polypeptides of 100 kD and 20 kD from the labeled translation products. Since the 20-kD component was also immunoprecipitated by the control antiserum, we concluded that the 100-kD polypeptide was the Sm-97 translation product and that intact message encoding the Sm-97 protein was therefore present in our mRNA preparation. The rabbit antibodies to Sm-97 were then affinity-purified on a SWAP immunoabsorbent column for use in immunoscreening of the cDNA library.

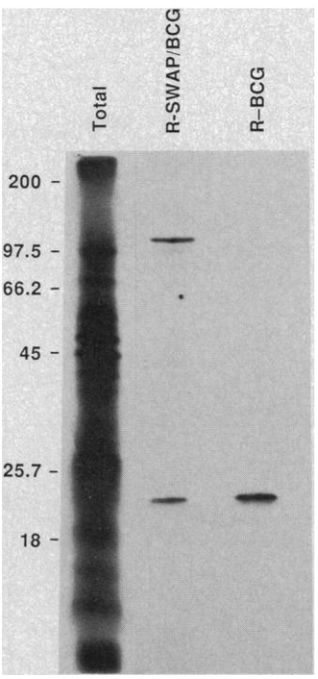


Fig. 1. Immunoprecipitation of in vitro translation products by rabbit antiserum to Sm-97. Adult schistosome poly(A)$^+$ mRNA was in vitro translated and immunoprecipitated as described (*12*) except that the translation products were absorbed with normal rabbit serum and Sepharose CL 4B–protein A before addition of specific antibody. Lanes: R-SWAP/BCG, rabbit antiserum to SWAP antigens affinity-purified by using IgG from mice immunized intradermally with SWAP plus BCG; R-BCG, control rabbit antiserum to SWAP antigens nonspecifically bound by using immunoabsorbent beads prepared with IgG from mice immunized intradermally with BCG alone (*13*).

For library construction, double-stranded cDNA was synthesized from poly(A)$^+$ mRNA by the method of Gubler and Hoffman (*14*), the ends were blunted with Klenow fragment, and the cDNA was then treated with Eco RI methylase. Eco RI linkers were added and cDNA greater than 500 bp was selected by Sephacryl S-1000 chromatography (*15*). The cDNA was ligated with λgt11 and the recombinants packaged as previously described (*12*). *Escherichia coli* strain Y1090 ($r^-m^+$) was infected with the phage from the unamplified library and the resulting plaques were screened with the rabbit antibodies to Sm-97. From the initial screening of $1 \times 10^5$ recombinants, two positive clones, MAC 97.1 and MAC 97.3, were selected and successfully recloned to purity. The identity of the expression products of these clones was confirmed by their reaction with a specific monoclonal antibody (Mab) to Sm-97, MBL-Sm-4B1 (4B1) (Fig. 2). Two other Mab's to Sm-97, MBL-Sm-1A6 (1A6) and MBL-Sm-4D1 (4D1), which recognize Sm-97 epitopes distinct from 4B1 (*9*), did not react with either the MAC 97.1 or MAC 97.3 expression products.

Further analysis of the expression products of the recombinants (Fig. 3) showed

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that MAC 97.1 produced a 170-kD fusion peptide with β-galactosidase that was recognized by both antibodies to β-galactosidase and by Mab 4B1. MAC 97.3 produced no detectable β-galactosidase epitopes, but did synthesize a peptide of about 52 kD that reacted with the Mab to Sm-97. Additional experiments showed that while fusion protein expression in MAC 97.1 was inducible by isopropylthio-β-galactoside (IPTG), the expression of the 52-kD polypeptide in MAC 97.3 was constitutive (*16*). These results suggested that in MAC 97.3 the insert was present in a reverse orientation with respect to the β-galactosidase promoter, and this was confirmed by subsequent sequence and mapping data (*17*). Correct expression of peptides from genes inserted in the reverse orientation in the λgt11 vector has been reported previously (*18*).

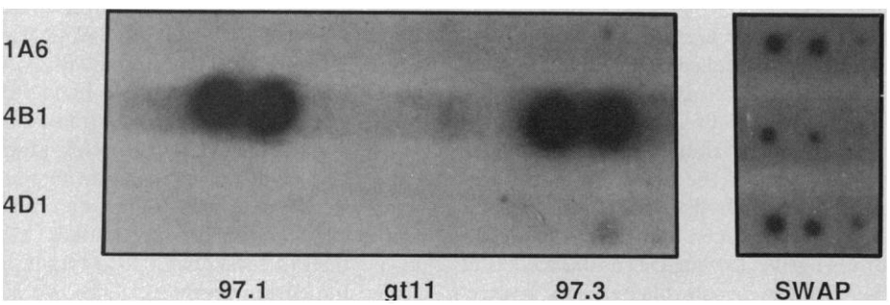


Fig. 2 (above). Recognition of recombinant clones by a monoclonal antibody to Sm-97. Approximately 1000 plaque-forming units of each phage (MAC 97.1, wild-type λgt11, or MAC 97.3) were spotted in duplicate on an agar plate containing *E. coli* Y1090. Plaque lifts were probed with a 1:2 dilution of hybridoma (1A6, 4B1, or 4D1) culture supernatant. Bound antibody was detected with $^{125}$I-labeled protein A. Strips of nitrocellulose containing spots of twofold dilutions of SWAP starting with approximately 1 μg were included in the same hybridization reactions.

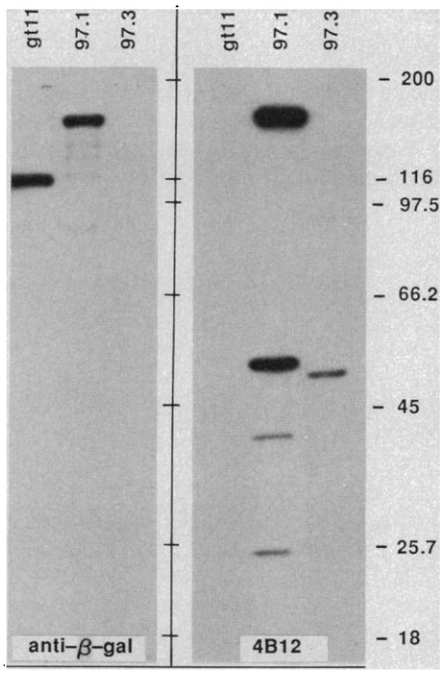


Fig. 3 (right). Western blot analysis of expression products of clones MAC 97.1 and MAC 97.3. Clones were grown as lysogens in *E. coli* Y1089 and separated by 7.5% PAGE-SDS as described (*12*). Filters were probed with either a 1:5000 dilution of rabbit antiserum to β-galactosidase or a 1:2 dilution of hybridoma 4B12 culture supernatant (clone 4B12 is a subclone of 4B1).

The complete sequence of the MAC 97.3 insert was determined by subcloning it, in both orientations, into m13mp18 and sequencing by the dideoxy-chain termination method (*19*) with subclones for sequencing produced by the Exo III procedure of Henikoff (*20*). Approximately 100 bp of the 5′ and 3′ ends of the MAC 97.1 insert were also sequenced in m13mp18 and shown to be identical to the ends of the MAC 97.3 insert. Additionally, the opposite or complementary single-stranded DNA's of the inserts from MAC 97.1 and MAC 97.3 in the m13mp18 vectors completely protected each other from nuclease S1 digestion, indicating that their nucleotide sequences were complementary along their whole length (*21*). Therefore, the inserts in both MAC 97.1 and MAC 97.3 consist of an identical 1317-bp sequence that translates in one reading frame only, with no termination signals, to give the amino acid sequence shown in Fig. 4B.

This amino acid sequence had distinct periodic heptad repeats $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$ with hydrophobic residues in positions a and d characteristic of an α-helical coiled-coil structure (*22*). A search of the Beckman MicroGenie Protein Bank (*23*) with the deduced amino acid sequence revealed a 36% homology with the amino acid sequence repeats of the α-helical coiled-coil rod region of the myosin heavy chain of the nematode *Caenorhabditis elegans* (*24*). On the basis of additional properties, however,

A

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1    GAAGAAATGA AGAGGAAATT CACTATGAGA ATTACCGAAC TTGAAGATAC TGCTGAAAGA
61   GAACGATTAA AAGCGGTATC ATTAGAAAAA CTTAAAACAA AATTAACATT AGAAATTAAA
121  GATTTACAAT CTGAAATAGA AAGTCTTTCA TTAGAAAATA GTGAATTAAT TCGTCGTGCT
181  AAAGCTGCTG AATCATTAGC TTCTGATTTA CAACGTCGTG TTGATGAATT AACAATTGAA
241  GTGAATACAT TAACATCACA AAATAGTCAA TTAGAAAGTG AAAATCTACG TTTAAAAAGT
301  TTAGTTAATG ATTTAACGGA TAAAAATAAT TTATTAGAAC GTGAAAATCG TCAAATGAAT
361  GGATCAAGTC AAAGAATTAA AAAGTTACTT CGTGATGCTA ATCGTCGTCT TACTGATTTA
421  GAAGCATTAA GATCGCAATT AGAGGCTGAA AGAGATAATC TTGCATCAGC TTTACATGAT
481  GCTGAAGAAG CATTACATGA TATGGATCAA AAGTATCAAG CATCACAAGC TGCATTAAAT
541  CATTTGAAAT CTGAAATGGA ACAAAGGCTT AGAGAAAGAG ATGAAGAATT AGAAAGTTTA
601  AGAAAAAGTA CTACTAGAAC AATTGAAGAA TTAACTGTTA CAATAACTGA AATGGAAGTT
661  AAATATAAAT CAGAATTATC ACGTTTAAAA AAACGTTATG AATCAAATAT TGCTGATTTA
721  GAAATTCAAC TTGATACAGC TAATAAAGCT AATGCAAATC TTATGAAAGA GAATAAAAAT
781  TTATCACAAC GTGTTAAAGA TTTAGAAACA TTTTTAGATG AAGAACGTCG TCTTCGTGAA
841  GCAGCTGAAA ATAATTTACA AATTACTGAA CATAAACGTT TACAATTAGC AAATGAAATT
901  GAAGAAATTC GTAGTACATT AGAAAATTTA GAACGTTTAC GTAAACATGC TGAAACAGAA
961  CTTGAAGAAG CTCAATCACG TGTTAGTGAA TTAACTATTC AACTTAATAT ATTAACTAAT
1021 GATAAACGTC GTCTTGAAGG TGATATTGGT GTAATGCAGG CTGATATGGA TGATGCTATT
1081 AATGCTAAAC AAGCTTCTGA AGATCGAGCA ATTAGATTAA ATAATGAAGT ATTACGTTTA
1141 GCTGATGAAT TACGTCAAGA ACAAGAAAAT TATAAACATG CTGAAGCATT AAGAAAACAA
1201 TTAGAAATTG AAATACGTGA AATTACAGTT AAATTAGAAG AAGCTGAAGC ATTTGCTACA
1261 CGTGAAGGTC GTCGTATGGT ACAAAAATTA CAGGCTCGTG TACGTGAACT TGAATCA
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B

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d e f g a b c d e f g a b c d e f g a b c d e f g a b c
*     *     *     *     *     *     *     *     *
  E E M K R K F T M R I T E L E D T A E R E R L K A V S      27
L E K L K T K L T L E I K D L Q S E I E S L S L E N S E     55
L I R R A K A A E S L A S D L L Q R R V D E L T I E V N T     83
L T S Q N S Q L E S E N L R L L K S L V N D L T D K N N L    111
L E R E N R Q M N G S S Q R I K K L L R D A N R R L T D     139
L E A L R S Q L E A E R D N L A S A L H D A E E A L H D     167
M D Q K Y Q A S Q A A L N H L K S E M E Q R L R E R D E E   196
L E S L R K S T T R T I E E L T V T I T E M E V K Y K S E   225
L S R L K K R Y E S N I A D L E I Q L D T A N K A N A N     253
L M K E N K N L S Q R V K D L E T F L D E E R R L R E A     281
A E N N L Q I T E H K R L Q L L A N E I E E I R S T L E N   309
L E R L R K H A E T E L E E A Q S R V S E L T I Q L N I     337
L T N D K R R L E G D I G V M Q A D M D D A I N A K Q A     365
S E D R A I R L N N E V L R L A D E L R Q E Q E N Y K H     393
A E A L R K Q L E I E I R E I T V K L E E A E A F A T R E   422
G R R M V Q K L Q A R V R E L E S                          439
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Fig. 4. (A) Nucleotide sequence of the insert of MAC 97.3. The insert is 1317 bp in length and is translated completely in one frame only to give the 439–amino acid sequence shown in (B). (B) The deduced amino acid sequence of MAC 97.3 showing the heptad repeat $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$ of amino acids arranged, according to the convention used for the myosin rod (*24*), of 28-residue amino acid regions and skip residues (amino acid numbers 196, 225, and 422). The "boxed" positions under "a" and "d" (asterisks) often contain hydrophobic residues whose side chains interlock in a regular pattern of "knobs-into-holes" packing to stabilize the two strands of an α-helical coiled-coil [see (*25*)].

we hypothesized that Sm-97 was not myosin but, in fact, paramyosin, an α-helical coiled-coil protein (*25*) that forms the core structure for myosin filaments in all invertebrates. To the best of our knowledge, the gene for paramyosin has never been cloned or sequenced.

Paramyosin has been implicated in the "catch" mechanism of muscle contraction, that is, the ability to sustain large muscle tensions with little energy expenditure, best exemplified in crab claw or oyster adductor muscles (*26*). For this reason a substantial amount of comparative research has been done on paramyosin from a large variety of invertebrate muscle. Comparison of the native Sm-97 protein and the deduced amino acid sequence of the cloned gene with properties of known paramyosins revealed compelling evidence that Sm-97 is paramyosin. The native protein, localized by indirect immunofluorescence to the muscle layer of the parasite (*9*), was distinct in molecular size from myosin heavy chain (≈200 kD) but equivalent to the size observed for paramyosins (≈100 kD) from seven phyla, including the platyhelminth *Hymenolepis diminuta* (*27*). During isolation of Sm-97 in the absence of protease inhibitors or EDTA the native polypeptide showed a proteolytic degradation pattern similar to that observed with known paramyosins, yielding molecules equivalent in size to β and γ paramyosins (*28*) [see (*9*) and Fig. 5]. In addition, the deduced amino acid sequence of clone MAC 97.3, which corresponds to about 50% of the total native protein, was similar in composition to known paramyosins but distinct from other myofibrillar proteins (*27, 29*) in having no proline or tryptophan, a high glutamic acid and glutamine composition (21.5%), and a lysine to arginine ratio <1 (0.66).

To confirm that the gene we had cloned encodes schistosome paramyosin we purified paramyosin directly from schistosomes (*30*) and compared it with Sm-97 and a known paramyosin isolated from the anterior byssus retractor muscle (ABRM) of the mussel *Mytilus edulis*. As shown in Fig. 5, rabbit antibodies to Sm-97 recognized the *Mytilus* paramyosin as well as Sm-97 in SWAP, Sm-97 affinity-purified from SWAP with the Mab 1A6 (*9*), and paramyosin purified biochemically from *S. mansoni* adult worms. It is interesting that neither antibodies from mice immunized intradermally with SWAP plus BCG nor the Mabs specifically recognizing epitopes on Sm-97 reacted with the molluscan paramyosin, but they did bind to schistosome paramyosin. This last difference probably reflects phylogenetic divergence in the paramyosins from these two species.

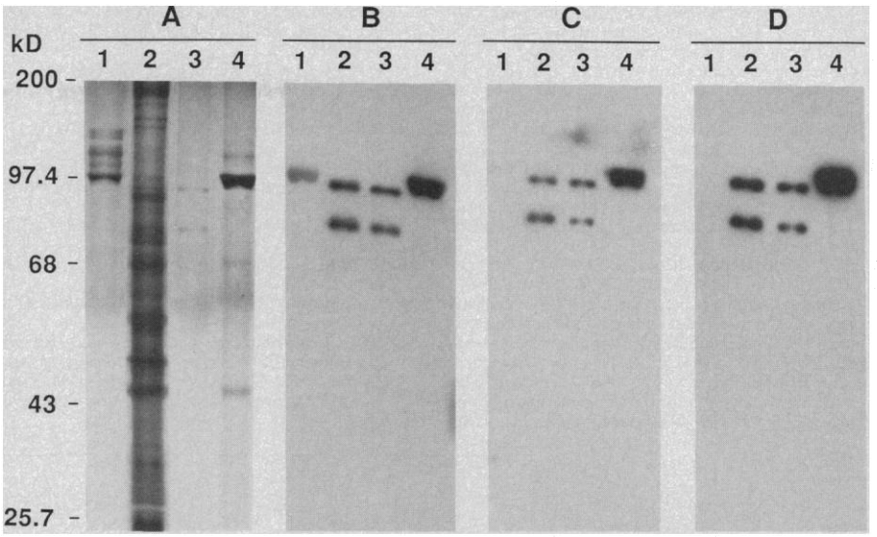


Fig. 5. Immunochemical comparison of Sm-97 with biochemically purified paramyosins. (A) A silver-stained gel of proteins separated by 7.5% PAGE. Lanes: 1, biochemically purified paramyosin from the ABRM of the mussel *Mytilus*; 2, SWAP prepared without protease inhibitors or EDTA; 3, affinity-purified Sm-97 from SWAP prepared without protease inhibitors or EDTA; 4, paramyosin purified biochemically from adult *S. mansoni*. (B, C, and D) Western blots of gels run in parallel to (A) and probed with different antibodies. (B) Polyclonal rabbit antiserum to affinity-purified Sm-97. (C) Antisera from mice immunized intradermally with SWAP plus BCG. (D) A 1:1 mixture of the hybridoma culture supernatants of Mab's 1A6 and 4B1.

Recent results indicate that affinity-purified expression products of both the MAC 97.1 and MAC 97.3 clones induce strong antibodies to Sm-97 when administered intradermally to mice in combination with BCG (*31*). Thus, the cloned portion of the molecule appears to contain some of the epitopes responsible for the immunogenicity of Sm-97 when presented by the intradermal route. Schistosome myosin and tropomyosin do not appear to have this property because antibodies are not made against them in our vaccine model (*9*). Therefore, the relevant epitopes are probably defined by paramyosin-specific amino acid sequences in Sm-97 and not by the α-helical conformation of the protein per se.

As an internally localized protein, Sm-97 is clearly distinct from the schistosomulum surface antigens previously identified as vaccine immunogens (*2–4*) and would not normally be available for direct attack by antibody, the mechanism of destruction classically hypothesized for the killing of schistosomes. Nevertheless, since the intradermal vaccine protocol appears to induce cell-mediated rather than humoral mechanisms of schistosome destruction (*1, 6, 8*), Sm-97 need not be surface-exposed to trigger the presensitized T lymphocytes that in turn activate macrophages for parasite killing. Presumably, this cell-mediated response could be stimulated by Sm-97 molecules or fragments released from parasites as a consequence of either normal protein metabolism or spontaneous parasite attrition.

In addition to its immunological potential as a vaccine immunogen, Sm-97 as paramyosin is of interest because of its probable physiological function for the parasite. As a core structure for myosin filaments, Sm-97 (paramyosin) may be an important molecular component in a "catch" mechanism aiding adult schistosomes in continuously maintaining themselves against the venule wall and thus avoiding dislodgment by the blood flow.

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13. Immunoglobulin G (IgG) from mice immunized intradermally with SWAP plus BCG by the intradermal route was isolated with protein A–Sepharose 4B and covalently linked to CNBr-activated Sepharose 4B. SWAP was added to the immunoabsorbent beads that were subsequently washed extensively. The beads containing bound Sm-97 were emulsified in Freund's complete adjuvant and injected into a rabbit. The rabbit was boosted three times with beads in Freund's incomplete adjuvant before blood was withdrawn. A control serum was prepared in a similar manner except that the IgG linked to the immunoabsorbent beads was from mice immunized intradermally with BCG only.
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EDTA. Approximately 0.4-ml fractions were collected and 2000 cpm from each fraction were analyzed by agarose gel and autoradiography. Fractions containing molecules between 500 and 5000 bp were pooled, the NaCl was adjusted to 200 m*M*, and the DNA was concentrated to 0.4 ml by passage through an Elutip-D cartridge (S&S) as described by the manufacturer. One microgram of dephosphorylated λgt11 (Promega Biotech) was added and the DNA precipitated at −20°C after the addition of 0.1 volume of 8*M* LiCl and 2.5 volumes of ethanol. The DNA pellets were redissolved in 20 μl of water and ligated.
16. D. E. Lanar, unpublished data.
17. A single Hind III site at position 1091 in the nucleotide sequence of the MAC 97.3 insert (see Fig. 4A) and four Hind III sites in the λgt11 vector [see R. A. Young and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1194 (1983)] allowed restriction mapping of the insert with respect to the vector. The mapping data were consistent with the inserts from MAC 97.1 and MAC 97.3 being in opposite orientations in λgt11; with the insert in MAC 97.1 being in the correct reading frame with the β-galactosidase promoter.
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32. We thank R. Doolittle for helpful discussions, E. D. Korn and J. A. Hammer III for first suggesting that Sm-97 might be paramyosin, J. E. Coligan for use of the MicroGenie software, and C. Cohen for the *Mytilus* paramyosin. This work was supported in part by grants from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and The Edna McConnell Clark Foundation.

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# Isolation of a New Virus, HBLV, in Patients with Lymphoproliferative Disorders

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**A novel human B-lymphotropic virus (HBLV) was isolated from the peripheral blood leukocytes of six individuals: two HTLV-III seropositive patients from the United States (one with AIDS-related lymphoma and one with dermatopathic lymphadenopathy), three HTLV-III seronegative patients from the United States (one with angioimmunoblastic lymphadenopathy, one with cutaneous T-cell lymphoma, and one with immunoblastic lymphoma), and one HTLV-III seronegative patient with acute lymphocytic leukemia from Jamaica. All six isolates were closely related by antigenic analysis, and sera from all six virus-positive patients reacted immunologically with each virus isolate. In contrast, only four sera from 220 randomly selected healthy donors and none from 12 AIDS patients without associated lymphoma were seropositive. The virus selectively infected freshly isolated human B cells and converted them into large, refractile mono- or binucleated cells with nuclear and cytoplasmic inclusion bodies. HBLV is morphologically similar to viruses of the herpesvirus family but is readily distinguishable from the known human and nonhuman primate herpesviruses by host range, in vitro biological effects, and antigenic features.**

THE IDENTIFICATION OF THE CAUSative agents (HTLV-I and HTLV-III) of two human hematopoietic cell diseases, adult T-cell leukemia (ATL) and acquired immune deficiency syndrome (AIDS), respectively, was possible because of the propagation of specific cell types in culture (*1–5*). Related retroviruses have been isolated from healthy Africans (HTLV-IV) and from some Africans with AIDS (LAV-2) (*6*). We have developed and utilized methods for activation and long-term cultivation of different types of fresh human lymphocytes, monocyte-macrophages, granulopoietic cells, and other cell types in liquid suspension culture for the purpose, in part, of finding new viruses.

In this study, cells primarily derived from fresh peripheral blood mononuclear cells from patients with associated lymphoproliferative disorders (Table 1) were grown in cell culture in RPMI 1640 supplemented with 20% fetal bovine serum and hydrocortisone at 5 μg/ml (*7*). In three instances (Table 1, patients 1–3), a small number of short-lived, large, refractile cells were observed in the primary cell cultures, which subsequently proved to be of value in predicting the presence of the new virus in other patients. The large cells were generally mono- or binucleated (Fig. 1a) and these cells frequently contained intranuclear and/or intracytoplasmic inclusion bodies. Electron microscopic examination (Fig. 2a) revealed a virus that we will call human B-lymphotropic virus (HBLV) (*8*). These large cells were also the only ones in culture that expressed viral antigens (described below) as measured by fixed-cell indirect immunofluorescence assays (IFA) (*9*). We also found a low number of these cells in fresh and/or cultured cells from three additional patients (Table 1, patients 4–6).

HBLV from the six patients was transmitted to freshly isolated phytohemagglutinin (PHA)-stimulated human leukocytes from umbilical-cord blood, adult peripheral blood, bone marrow, and spleen. After infection in vitro large refractile cells, similar to those noted in primary cultures, appeared within 2–4 days (Fig. 1b). These cells may initially have a growth advantage because they eventually became the predominant cells in the culture, surviving for an additional 8 to 12 days while other cells in the culture rapidly died. As in primary cell cultures, these large cells expressed viral nucleic acids as shown by in situ hybridization (Fig. 3) (*10*), and viral antigens as detected by IFA (Fig. 4, a and b). Virus production was confirmed by electron microscopy (Fig. 2b) (*8*).

The main features of this virus are: (i) uniform shape and size of virion, (ii) abundant extracellular virus production by infected cells, (iii) absence of perichromatin-like granules in the nucleus, (iv) absence of any cytoplasmic aggregates (the inclusion bodies of HBLV-infected cells are not virus clusters although dispersed single virus particles are present in the cytoplasm), (v) icosahedral

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