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Hybridoma antibody immunoassays for the detection of parasitic infection: further studies on a monoclonal antibody with immunodiagnostic potential for schistosomiasis japonica

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Summary

The binding of a hybridoma-derived antibody (designated IPH.134-18-6) to an extract of the adult worm of *Schistosoma japonicum* has been further characterized. This antibody has immunodiagnostic potential for detection of infection with *S. japonicum* in the Philippines since the binding of labeled hybridoma antibody to a crude adult worm extract in a solid-phase radioimmunoassay (RIA) is inhibited by sera from >90% of known infected individuals, and no false positive reactions have yet been observed. Moreover, most patients with high fecal egg outputs or prominent disease have high serum inhibitory activity. All evidence indicates that IgG antibodies are the serum inhibitors in the competitive RIA. By estimating the amount of inhibitory activity (antibody?) of IPH.134-18-6 type in sera of infected patients and animals, the conclusion was reached that the antigen to which this hybridoma is directed is a strong immunogen. Moreover, no evidence of genetic unresponsiveness was obtained in strains of inbred mice infected with *S. japonicum*. IPH.134-18-6 does not bind to *S. japonicum* egg extracts. However, the actual stage and species specificity of this antibody has not yet been determined unequivocally and must await the screening of sera from individuals with monospecific *S. mansoni* and *S. hematobium* infections as well as assays using schistosomules, male and female adult worms and juveniles.

Key words: hybridoma (monoclonal) antibody; *Schistosoma japonicum*; immunodiagnostic reagent; mice; humans; Philippines.

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Introduction

In immunoparasitology, hybridoma-derived (monoclonal) antibodies are being used as probes for antigenic determinants, the development of immunodiagnostic tests (IDTs) of high specificity, and the analysis of antibody-mediated parasite-inhibitory effects in vivo and in vitro (reviewed in Mitchell and Cruise, 1981). Recently, a murine hybridoma was developed which secretes an antibody with immunodiagnostic potential for schistosomiasis japonica in man (Mitchell et al., 1981a). The binding of the hybridoma-derived antibody (designated, in abbreviated form, IPH.134) to a crude *Schistosoma japonicum* adult worm extract was inhibited by sera from 40 out of 44 known infected patients in the Philippines but not by sera from individuals in the Philippines or neighboring countries with a variety of other parasitic infections, including trematode infections. With the schistosomiasis sera examined, most (8 out of 9) patients with high egg counts in feces (>300 eggs per gram) had high inhibitory titers in sera. Moreover, 3 patients known to have prominent splenomegaly and hepatomegaly had high serum inhibitory titers in the competitive radioimmunoassay. The results raise the possibility that an IDT based on the antigen to which the IPH.134 antibody is directed may provide an indication of the level of infection or disease status in individuals. Such a test would be of great value in the monitoring of control programs and in the identification of individuals at risk in terms of development of disease. In this paper we report results of further experiments with this antibody, and claims made regarding its immunodiagnostic potential are substantiated.

Materials and methods

Hybridoma IPH.134

Full details have been provided on the production and selection of the IgG_{2a}-secreting hybridoma cell line, IPH.134 (or more precisely, the double cloned, IPH.134-18-6) using cells from antigen-injected mice and the modified myeloma cell line NS-1 (Mitchell et al., 1981a; Mitchell et al., 1979). BALB/c mice were injected with lyophilized mouse-derived *S. japonicum* adult worms or their aqueous extracts in Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan, USA) and boosted without adjuvant. Spleen cells for fusion were taken at 4 days after the last antigen injection and IPH.134 (and other antibody-secreting cell lines) selected using an aqueous adult worm extract (AWE) prepared from homogenized and sonicated lyophilized *S. japonicum* worms in a solid-phase radioimmunoassay (RIA). The AWE was processed to remove any immunoglobulin (Ig) binding moieties by passing it through a mouse Ig-Sepharose column (Mitchell et al., 1981a). The IPH.134 protein was purified from bulk culture supernatants (or ascites fluids from pristane-injected and tumor-bearing mice) using *Staphylococcus aureus* protein A-Sepharose as described previously (Mitchell et al., 1979). The protein was labeled with ¹²⁵I using the chloramine T method to a specific activity of approximately 370 kBq/ μ g (10 μ Ci/ μ g) by John Pye of this Institute.

Sera

Full details of the two series of human schistosomiasis sera (consisting of 19 and 20 samples) used in the present experiments have been reported elsewhere (Mitchell et al., 1981a; Tapales et al.,

1981). They were obtained from individuals presenting at the Institute of Public Health, Manila, Philippines; in all patients, eggs were detected in feces and circumoval precipitating (COP) antibodies were detected in sera. The 19 sera in one series were shown to contain anti-egg antibodies as detected in a solid-phase RIA using extracted *S. japonicum* egg antigen (EA) (Tapales et al., 1981). Other human sera from Philippines, Papua New Guinea, The People's Republic of China and Melbourne were also available (Mitchell et al., 1981a).

Sera were obtained from mice taken from the Melbourne Laboratory to Manila where they were infected percutaneously with 25 cercariae of *S. japonicum* (Mitchell et al., 1981b). Serum from an infected rabbit was obtained from Dr. Huang Sung-Ru of the Friendship Hospital, Beijing, People's Republic of China.

Solid-phase competitive RIA

An amount of stock *S. japonicum* processed AWE in borate buffer pH 9.5 was chosen for coating to polyvinylchloride microtiter plates (Dynatech Laboratories, Alexandria, Va., USA) which resulted in >50% but <100% of the plateau level of binding of 20,000 cpm of ¹²⁵I-IPH.134. Antigen in 50 μ l was added to the plate, left for 3–4 h at room temperature in a humidified box, unbound material removed and 50 μ l 0.5% bovine serum albumin (BSA) (Fraction V, Armour Pharmaceutical Co., Eastbourne, England) in mouse tonicity phosphate buffered saline pH 7.3 (PBS) added to each well. After 1 h, plates were rinsed in 0.05% Tween 20 in PBS, approximately 20,000 cpm of ¹²⁵I-IPH.134 in 25 μ l and 25 μ l of a titration of inhibitor (usually serum and commencing at 1:20 dilution) in 0.05% Tween 20 and 0.5% BSA in PBS added to each well and left overnight at room temperature. Radioactivity bound to individual wells was determined in an autogamma counter after rinsing the plates and cutting with a hot wire (Mitchell et al., 1979; Craig et al., 1980).

In those assays in which plates were coated with IPH.134, 10 μ g/ml protein was used and a titration of human serum added after residual binding sites on the plate were saturated with BSA. This was left for 4 h, unbound material washed from the plates, and 20,000 cpm of ¹²⁵I-sheep anti-human Ig which had been affinity purified (Tapales et al., 1981) and absorbed with mouse IgG and IgM, added to each well, left overnight and bound radioactivity determined as above.

Results

Summary of results using ¹²⁵I-IPH.134 and processed S. japonicum AWE in a competitive RIA with human sera

Of several hybridomas which secreted antibodies to a *S. japonicum* AWE, one (IPH.134-18-6) had immunodiagnostic potential. Thus the binding of ¹²⁵I-IPH.134-18-6 to processed *S. japonicum* AWE could be inhibited by sera from >90% Philippine patients known to have *S. japonicum* infection and no false positive reactions were obtained (Fig. 1). The other aspect of the data which is apparent in Fig. 1 is that, at a serum dilution of 1:320 (taken from a full titration curve), the majority of patients with high fecal egg outputs, or known prominent disease, had high levels of inhibitory activity in serum. Evidence has been presented elsewhere that IgG antibodies (rather than immune complexes, free parasite antigen or antiidiotypic antibodies) are responsible for serum-mediated inhibition in the competitive RIA (Mitchell et al., 1981a, and see below).

Concerning the parasite life-cycle stage specificity of the target antigen of the IPH.134 antibody, the evidence that the antigen is confined to, or at least

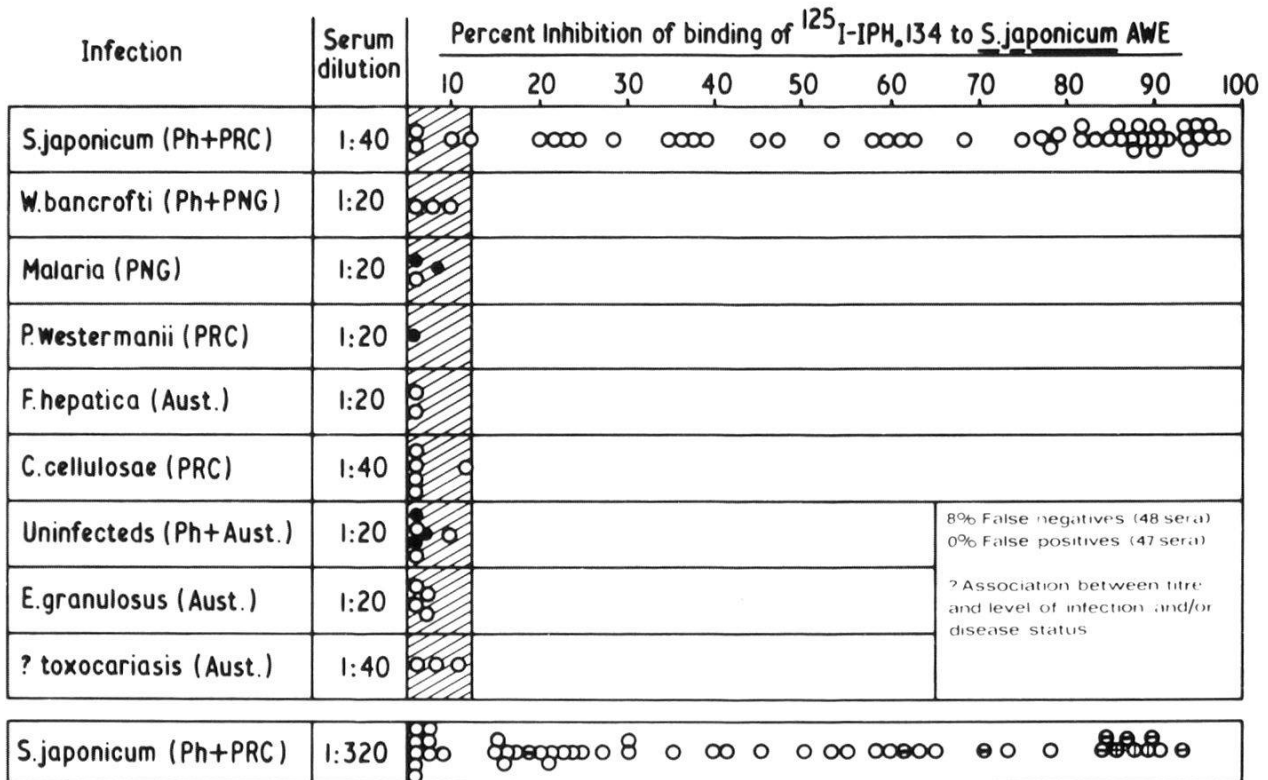


Fig. 1. Summary of results using a competitive radioimmunoassay with *S. japonicum* AWE, the labeled hybridoma antibody IPH.134-18-6, and various sera from Australia (Aust.), Philippines (Ph), People's Republic of China (PRC), and Papua New Guinea (PNG). Individual sera represented by open circles, pooled sera by closed circles, and patients with high fecal egg counts (\ominus) and known prominent disease (\oplus) are also indicated. From the titrations performed with these various positive and negative sera (Mitchell et al., 1981a) a serum dilution of approx. 1: 50 will differentiate (with < 10% false negatives) between the background inhibition with high amounts (low dilutions) of non-positive sera and the lowest of the positive schistosomiasis japonica sera. Moreover dilutions of 1: 500 and 1: 5000 will be capable of detecting sera with high inhibitory activity.

better expressed in, adult worm extracts (AWE) versus an egg extract (EA) is presented in Fig. 2. The EA used in this study was an homogenized and sonicated preparation from lyophilized *S. japonicum* eggs used previously for analysis of anti-egg antibody responses in *S. japonicum*-infected individuals using a solid-phase RIA (Tapales et al., 1981). This EA was compared with both processed and unprocessed *S. japonicum* AWEs (see Materials and methods) in assays with two labeled hybridoma antibodies: viz., ¹²⁵I-IPH.134-18-6, and an IgG₁ antibody selected for its capacity to bind to EA (i.e. ¹²⁵I-SEF.85-5-3). It can be seen in Fig. 2 that ¹²⁵I-IPH.134 bound to the AWEs but not to EA and that ¹²⁵I-SEF.85 bound better to EA than the AWEs. The latter will contain EA since the adult worms used for AWE preparation include egg-laying females. (The relatively low binding of ¹²⁵I-SEF.85 to all antigen preparations in Fig. 2 probably reflects damage to the hybridoma antibody resulting from iodination as this low level binding is not seen with unlabeled hybridoma antibody plus ¹²⁵I-labeled anti-mouse Ig [unpublished results].)

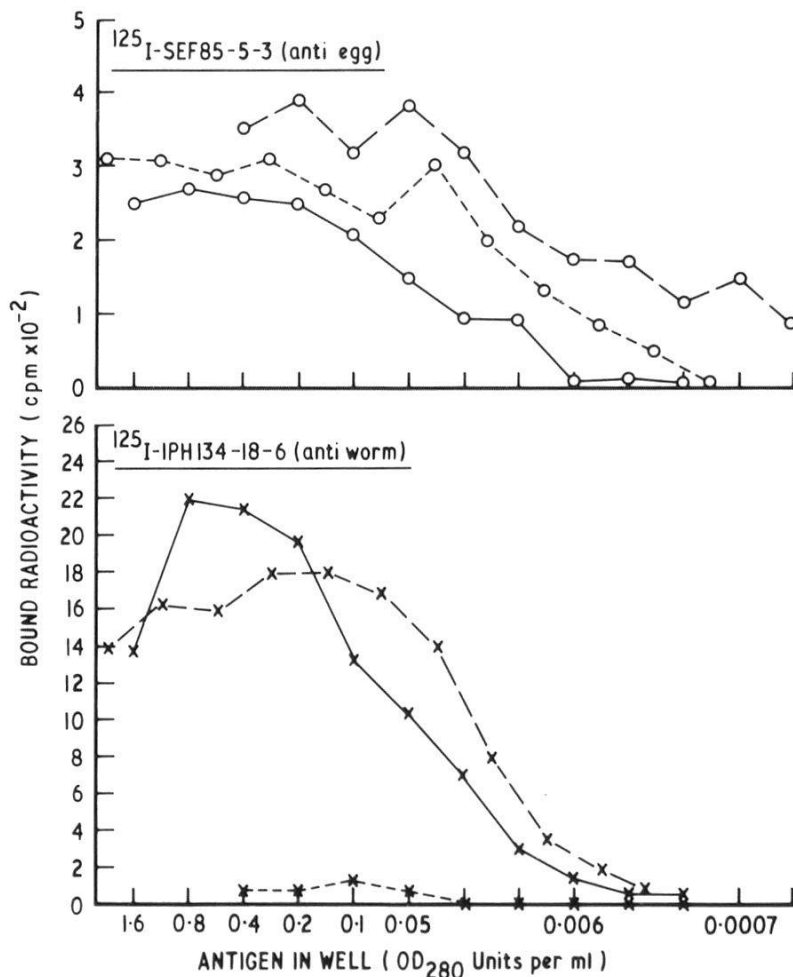


Fig. 2. Binding of two IgG hybridoma antibodies, IPH.134 (x) and SEF.85 (o) to *S. japonicum* processed AWE (—), unprocessed AWE (x—x, o-----o) and a *S. japonicum* egg extract (x-----x, o—o) in solid-phase RIAs. The highest concentration of adult worm antigen (OD₂₈₀ of 1.6) is equivalent to approximately 100 μg protein as determined by the method of Hartree (1972).

An estimation of the amount of IPH.134-type inhibitory activity in sera from infected mice, humans and a rabbit

In Fig. 3, a curve for the inhibition of binding of $^{125}\text{I-IPH.134-18-6}$ is shown using cold protein A-purified IPH.134-18-6 as the inhibitor. From the curve, and choosing a conservative figure, 4 ng cold IPH.134 results in 50% inhibition of binding in the assay. Using this figure, and ignoring combining-site affinity considerations in the calculation, the serum from a 45-year-old infected female with approximately 3000 eggs per gram (epg) feces (serum No. 27), one 16-year-old infected male with approx. 500 epg feces (serum No. 5), and one 50-year-old infected female with <100 epg feces (serum No. 10), contain approx. 0.8 mg/ml, 0.6 mg/ml and 0.025 mg/ml of inhibitory IPH.134-like activity (presumably antibody) in their sera, respectively. Moreover, serum from a 37-day-infected rabbit, one pool of protein A-binding Ig from the sera of 40-day-infected C57BL/6 mice (see below), and the pooled ascites fluids of IPH.134 tumor-bearing mice contain approx. 0.025 mg/ml, 0.05 mg/ml and 2.5

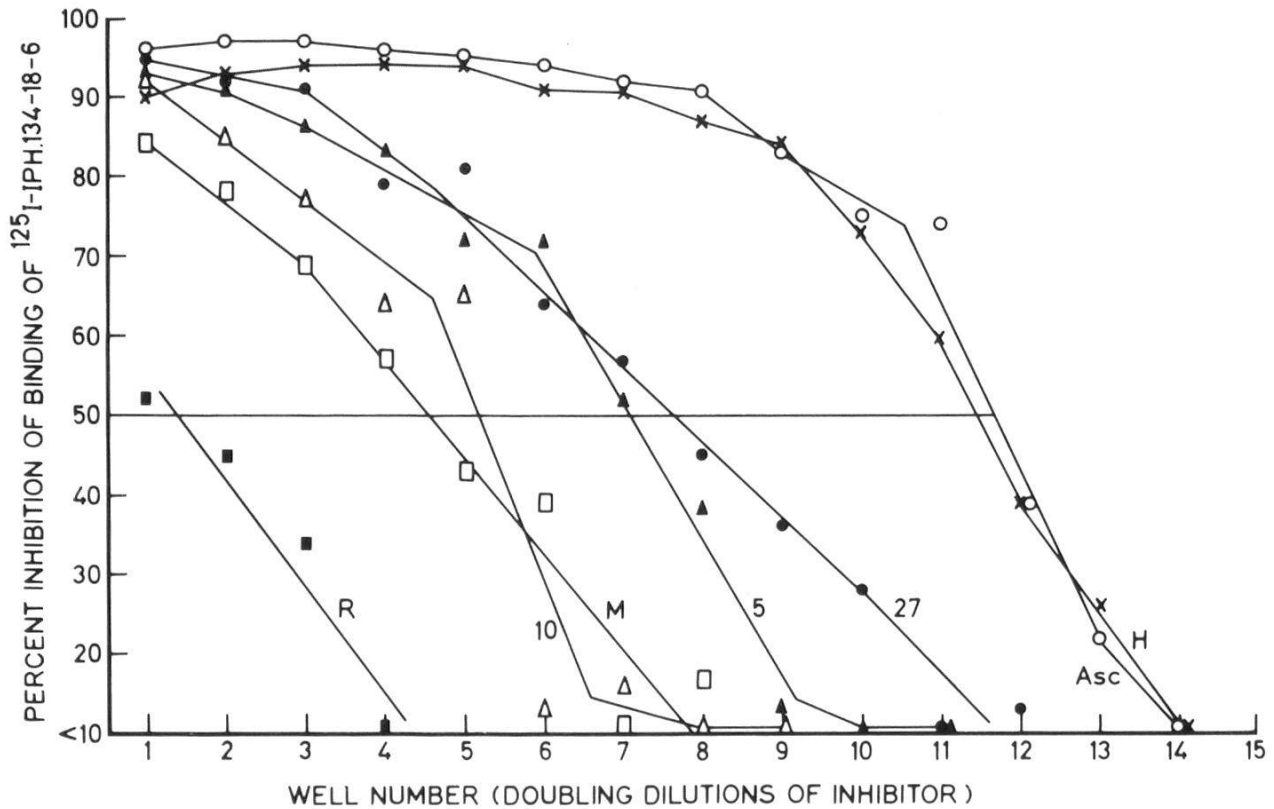


Fig. 3. Inhibition of binding of 2 ng ^{125}I -IPH.134-18-6 to *S. japonicum* AWE using various inhibitors in 25 μl doubling dilutions from well No. 1 of 0.5 mg/ml bulk culture-derived and protein A-purified IPH.134-18-6 (x—x; H), 1:10 dilution of ascites fluid from IPH.134-18-6 tumor-bearing mice (o—o; Asc), 1:50 dilution of No. 27 human serum (●—●; 27), 1:50 dilution of No. 5 human serum (▲—▲; 5), 1:10 dilution of No. 10 human serum (Δ — Δ ; 10), the equivalent of 1:32 serum dilution of protein A-purified Ig from a pool of sera from 40-day-infected C57BL/6 mice (\square — \square ; M), and a 1:100 dilution of serum from a 37-day-infected rabbit (\blacksquare — \blacksquare ; R). With the conservative assumption that 4 ng protein A-purified hybridoma antibody in 25 μl results in 50% inhibition and ignoring affinity considerations, the amounts of inhibitory antibody in the sera compute out at approximately 0.8 mg/ml for serum No. 27, 0.6 mg/ml for serum No. 5, 2.5 mg/ml for the IPH.134 mouse ascites fluid, 25 μg /ml for serum No. 10, 50 μg /ml for the infected C57BL/6 serum pool and 25 μg /ml for the infected rabbit serum.

mg/ml of IPH.134-type inhibitory activity. The figures for sera, or purified Ig, even if overestimated by a factor of 10 or more, indicate that the *S. japonicum* adult worm antigen to which the IPH.134 monoclonal antibody is directed is a powerful immunogen in infected animals and man.

*Inhibition of binding of ^{125}I -IPH.134 to processed *S. japonicum* AWE using sera from infected mice*

During studies on the susceptibility of different strains of mice to *S. japonicum* infection and differences in immune responses in infected mice, two strains of mice were identified as being different from others. CBA/H mice appeared to be low COP anti-egg antibody producers and 129/J mice were relatively resistant to infection (Mitchell et al., 1981b). The presence of genetic nonresponsive-

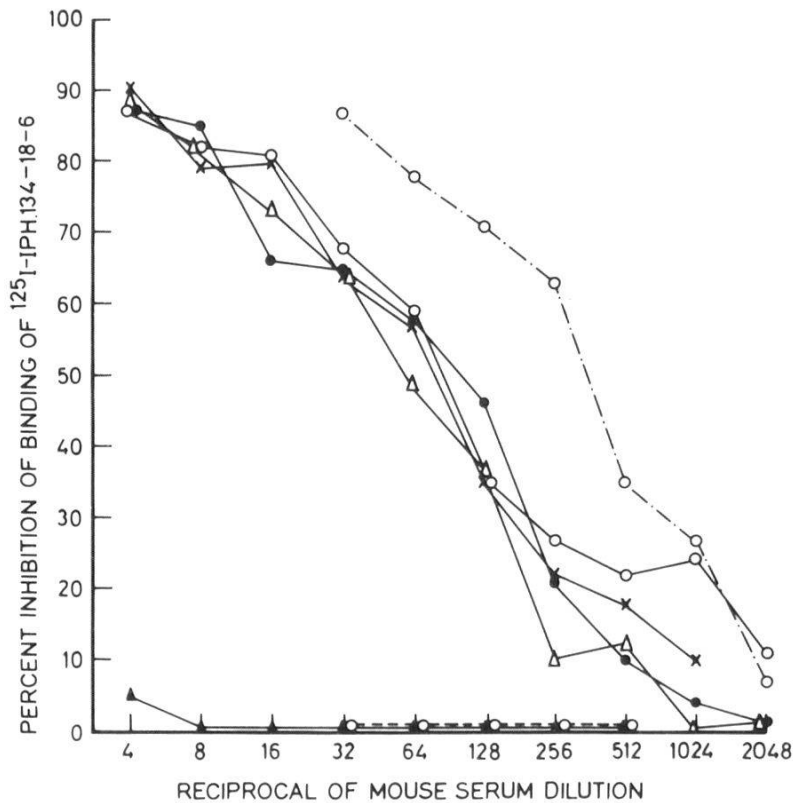


Fig. 4. The inhibition of binding of ^{125}I -IPH.134-18-6 to *S. japonicum* AWE using serum pools from mice infected for 40 days with *S. japonicum*: (x—x) sera from CBA/H mice, (o—o) C57BL/6 mice, (●—●) BALB/c mice, (Δ — Δ) 129/J mice, (\blacktriangle — \blacktriangle) BALB/c mice chronically infected with *Leishmania tropica*, and the protein A-Sepharose run through (o-----o) and pH 3 eluate (o-----o) of infected C57BL/6 serum concentrated down to the volume equivalent of serum loaded on to the 5 ml protein A-Sepharose column.

ness in hosts to the antigenic determinant to which IPH.134 is directed would militate against the utility of this antigen in an IDT. Four pools of sera from 40-day-infected CBA/H, BALB/c, C57BL/6 and 129/J mice were available for testing whether mouse strains differed in response to the IPH.134 antigen. At the time of bleeding (day 40), CBA/H mice contained 16.0 ± 2.2 worms (arithmetic mean \pm SEM) with approx. 600 epg feces; C57BL/6 mice: 13.5 ± 2.0 worms and 600 epg feces; BALB/c mice: 8.9 ± 1.7 worms and 300 epg feces; 129/J mice: 2.4 ± 1.6 worms and 90 epg feces. As can be seen in Fig. 4 all mouse strains responded by production of inhibitory serum activity. An important control serum pool from *Leishmania tropica*-infected BALB/c mice which, like sera from *S. japonicum*-infected mice will be hypergammaglobulinemic (Chapman et al., 1979), was without inhibitory activity. Moreover, when pooled sera from the infected C57BL/6 mice were fractionated on protein A-Sepharose, all the inhibitory activity was in the IgG fraction eluted at pH 3.0 with none in the run-through (non-binding) fraction (Fig. 4). The data on sera from infected mice is encouraging in terms of the absence of genetically based nonresponsiveness in at least 4 strains of the "permissive" host, the mouse.

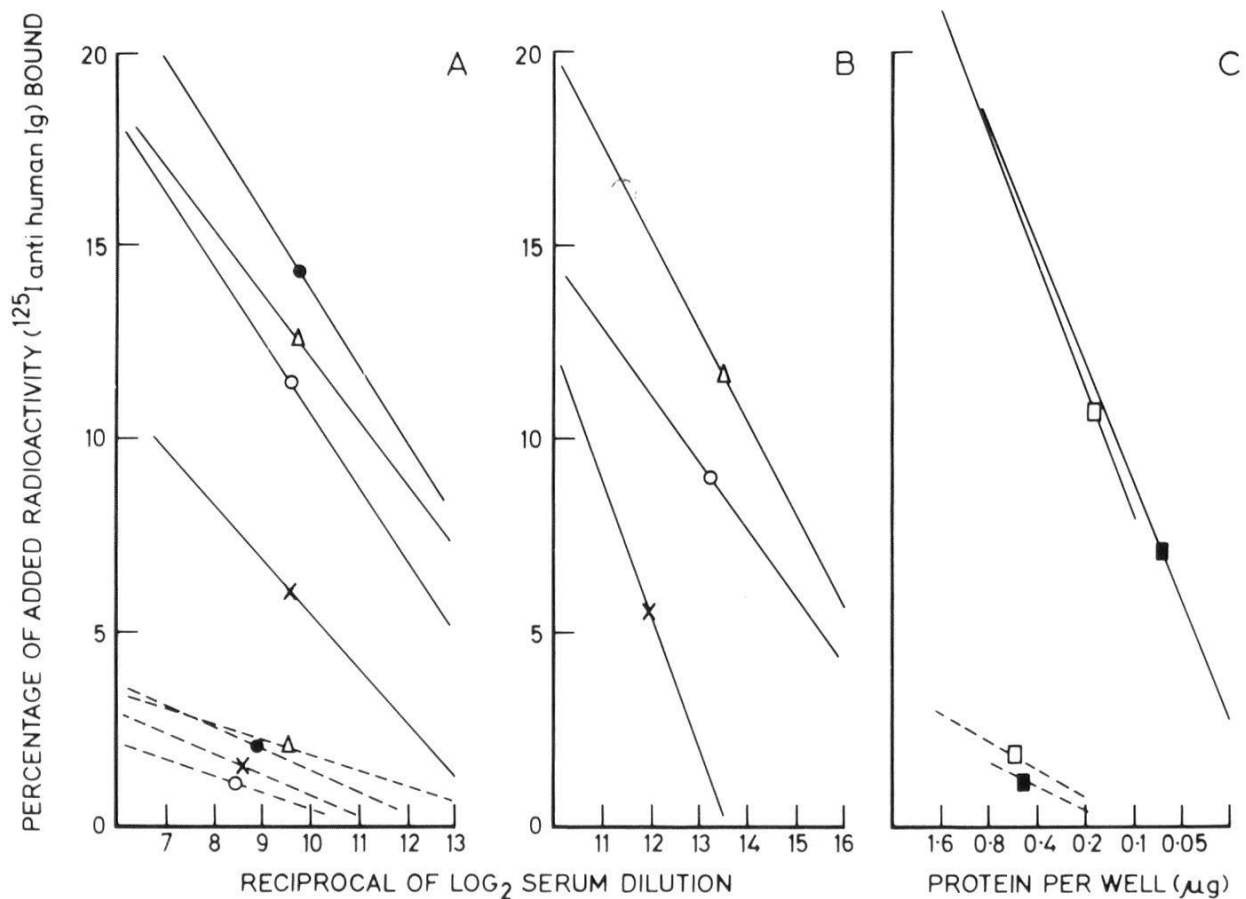


Fig. 5. Binding of ^{125}I -labeled sheep anti-human Ig (absorbed against mouse IgG and IgM) to plates coated with $10\ \mu\text{g}/\text{ml}$ IPH.134 (Panels A and C) or a control IgG_{2a} hybridoma antibody (Panel B) and reacted with sera from an uninfected (x = No. 6) or three *S. japonicum*-infected human sera (● = No. 3 with high hybridoma inhibitory serum titer and high fecal egg count; △ = No. 1060 with high hybridoma inhibitory serum titer and intermediate fecal egg count; ○ = No. 938 with high hybridoma inhibitory serum titer and low fecal egg count) or protein A-Sepharose purified Ig from two infected individuals with high (No. 27, □) or low (No. 26, ■) hybridoma inhibitory serum titers. Binding of radioactivity to plates not coated with hybridoma antibody (but coated with BSA) is indicated by the dashed lines. Details of sera used are provided in Mitchell et al. (1981a).

Further studies on the nature of inhibitory activity in infected human sera

Previous results using protein A-Sepharose and Sephacryl SF-300 fractionation of human sera, strongly suggested that IgG antibodies were responsible for inhibition of binding of ^{125}I -IPH.134 in the competitive RIA. Moreover no binding of ^{125}I -IPH.134 to infected human sera could be detected when the latter were coated to PVC plates (Mitchell et al., 1981). In further studies to determine whether immune complexes (containing antigen to which IPH.134 is directed) or antiidiotypic (anti-Id) antibodies were present in sera from infected individuals, PVC plates were coated with IPH.134 or another IgG_{2a} hybridoma antibody (with specificity for azobenzene arsonate, a gift from G. Morahan). Dilutions of human sera were added, and an ^{125}I anti-human Ig, absorbed against mouse Ig, used as the radioactive read-out reagent. It can be seen in Fig. 5 that: (1) Ig in human sera bound preferentially to plates coated with

IPH.134 (versus BSA), (2) Ig in uninfected human serum bound although to a lesser extent than 3 sera (Nos. 3, 938 and 1060) of high inhibitory titer in the competitive RIA, (3) the same ranking of binding activity was found when the plates were coated with a control IgG_{2a} hybridoma antibody rather than IPH.134, and (4) protein A-purified IgG fractions of sera Nos. 27 and 26 which have at least a 50 × difference in inhibitory titer and a 5 × difference in amounts of protein A-binding Ig (Mitchell et al., 1981a), had no difference in binding activity to IPH.134 on the plate when equivalent amounts of protein were assayed. The results probably reflect the activity of natural anti-mouse Ig antibodies in human sera (perhaps related to rheumatoid factors) or other mouse Ig:human Ig interactions detected in this very sensitive assay. The 3 infected human sera, Nos. 3, 938 and 1060, like serum No. 27, can be expected to be hypergammaglobulinemic and therefore to have higher binding activity than uninfected human serum. Thus, the assay failed to detect the presence of specific anti-Id antibodies (directed against IPH.134) or immune complexes (containing the antigen to which IPH.134 is directed). The results of these experiments demonstrate how critical it is to control for apparent nonspecific binding effects when using hybridoma antibodies in solid-phase for the detection of anti-Id antibodies or immune complexes.

Discussion

In this paper, we have described in further detail the characteristics of an IgG_{2a} anti-*S. japonicum* hybridoma-derived antibody, designated IPH.134, which has immunodiagnostic potential for schistosomiasis japonica infection in the Philippines. An estimation has been made of the amount of IPH.134-like inhibitory activity (and presumably antibody, see below) in sera from selected Philippine schistosomiasis patients, infected mice and a rabbit. The figure of close to 1 mg/ml for serum from a patient with high fecal egg counts suggests that the antigen to which IPH.134 is directed is a potent immunogen in man. Moreover, if this is in any way an accurate assessment of the level of antibody to a *single* antigenic determinant of *S. japonicum*, then the marked hypergammaglobulinemia in patients with high fecal egg counts (Mitchell et al., 1981a) may consist of a high proportion of anti-*S. japonicum* antibodies and not nonspecific immunoglobulins.

Mice were also responders at day 40 of infection (following exposure to 25 cercariae) in that sera inhibited the binding of ¹²⁵I-IPH.134 to AWE. However, no ranking of RIA hybridoma inhibitory titers in serum pools was noted between mice with high (CBA/H, C57BL/6), intermediate (BALB/c) and low (129/J) adult worm numbers and fecal egg counts (Fig. 4). We have emphasised previously that the solid-phase RIA as an IDT may provide information on the level of infection in man and/or disease status which would be of enormous value in epidemiological studies as well as for immunodiagnosis in the individ-

ual patient. The result obtained with mouse serum does not really bear on possible correlation between worm burden and inhibitory titer in long-term infected humans. If worms are being destroyed just prior to day 40 in 129/J mice then serum inhibitory antibody titers may not be very different between mice of this strain and others with more persistent worm burdens. Current experiments have been designed to determine the stage of the life cycle at which resistance in 129/J mice is expressed. In addition, sera from long-term, chronically infected mice are being obtained, these sera being more relevant to the situation pertaining in man with years of exposure to *S. japonicum*.

A feature of the RIA hybridoma inhibitory test was that CBA/H mice were found to be high responders quite unlike the situation in response to *S. japonicum* egg antigens (Mitchell et al., 1981b). The preliminary finding that CBA/H mice are low COP antibody responders (cf. 11 other strains) raises the possibility that genetic variability in host responsiveness could account for a low proportion of false negatives in the COP test for schistosomiasis japonica. Nevertheless in both *S. japonicum* and *Schistosoma mansoni* infections, the high specificity and sensitivity of the COP test have been proven (e.g. Garcia et al., 1968; Yogore et al., 1978; Hillyer et al., 1979). The major difficulties with the test are standardization of eggs to be used, the difficulties of performing quantitative assays for antibody, and the lack of information obtained from the test on presumed worm burdens. Using a RIA with egg antigens, infected teenagers were found to be higher responders than infected older individuals (Tapales et al., 1981). This difference in titer according to age (rather than presumed infection level or disease status) was not seen in the RIA hybridoma inhibitory assay (Mitchell et al., 1981a).

There is considerable evidence that IgG anti-*S. japonicum* antibodies are the inhibitors in the RIA hybridoma inhibitory assay with human sera (Mitchell et al., 1981a). ¹²⁵I-IPH.134 does not bind to infected human serum coated on to plates and, using Sephacryl SF-300 chromatographic fractionation of infected human serum, inhibitory activity in the RIA coincides precisely with the large IgG peak with nothing detectable in the IgM region (or the area between the IgM and IgG regions) of the protein profile. In the present series of experiments it was shown that inhibitory activity in infected C57BL/6 serum bound to protein A-Sepharose suggesting that IgG is responsible (Ey et al., 1978; McKenzie et al., 1978). No evidence for antiidiotypic antibodies or immune complexes involving the IPH.134 hybridoma antibody specificity or its target antigen was obtained using various infected human sera with different inhibitory titers in a binding RIA with IPH.134 on the plate and an ¹²⁵I anti-human Ig as the readout (Fig. 5).

To facilitate field application of the IDT, attempts to isolate the antigen to which IPH.134 is directed are in progress. With purified antigen (or the antigenic determinant) on the plate, the enzyme-linked immunosorbent assay (ELISA) with enzyme-linked anti-human Ig may then be used. In the expectation that

large amounts of antigen may be difficult to obtain we are making attempts to use murine antiidiotypic antibodies directed against the IPH.134 antibody in lieu of the antigen in an ELISA assay. However, no encouragement to persist with this approach has been obtained to date using solid-phase, affinity-purified anti-Id antibodies (prepared from pooled mouse sera) and sera from infected humans for inhibition of binding of ^{125}I -IPH.134 (see also Mitchell et al., 1979).

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