EFFECTS OF IRRADIATION AND TUNICAMYCIN ON THE SURFACE GLYCOPROTEINS OF   **MR-18**
Schistosoma mansoni

J.R. Kusel, A. Wales, *L. Vieira and K-Y Wu

University of Glasgow and *Universidade Federal de Minas Gerais, Belo
Horizonte, Brazil

INTRODUCTION

Some of the functions of glycoproteins in parasite surface may be
(a) to act as receptors for growth substances (b) to act as a physical or
immunochemical barrier to cells and antibodies of the host's immune system
or (c) to maintain the structure of the surface membrane. Glycoproteins
also act as potent immunogens in human and experimental hosts. Antibodies
can be induced against both polypeptide and carbohydrate epitopes in a
natural infection while heavily gamma or ultra-violet irradiated cercariae
appear to stimulate antibodies recognising polypeptide epitopes (Pirlanta
Omer-Ali Magee, Kelly and Simpson 1986). It would appear that irradiation
alters the parasite so that glycoproteins are presented to the host's
immune system in a modified form. In this paper we have examined the
distribution of a variety of glycoproteins on the surfaces of normal
cercariae and schistosomula and studied the changes in this distribution
after irradiation with ultra-violet light, and treatment with tunicamycin,
an inhibitor of glycosylation (Elbein, 1987). We speculate that inhibition
of glycosylation can alter the processing and presentation of surface and
secreted glycoproteins, and is in part responsible for the altered
immunogenicity of irradiated cercariae.

MATERIALS AND METHODS

Glycocalyx and snail tissue preparations

Glycocalyx from cercariae was obtained during the mechanical
transformation of cercariae into schistosomula as previously described
(Vieira et al., 1986). Cercarial released products containing glycocalyx
were obtained in two fractions: one after the 4^oC vortex step (G1) and the
other after incubation of cercarial bodies for 90 minutes at 37^oC.
Preparations of these fractions for injection in rabbits was done as
described previously (Vieira et al., 1986). Snail haemolymph was obtained
by bleeding non-infected Biomphalaria glabrata by heart puncture. Soft
tissues were obtained by removing the shell from bled snails, cutting the
muscular foot out and homogenizing the remaining body with saline. Both
preparations were centrifuged at 1000g at 4^oC for 1 hour, for removal of
large tissue debris. Protein was assayed by the Lowry method.

Antisera

Antisera anti-glycocalyx 1, (anti-G1), anti-glycocalyx 2 (anti-G2),
anti-haemolymph (anti-HM) and anti-snail soft tissues (anti-ST) were

obtained in New Zealand rabbits as described by Johnstone & Thorpe (1985). Before immunization rabbits were bled from the ear, in order to obtain their pre-immunization sera (normal rabbit sera). The rabbits were then injected with 500µg of protein of the antigens diluted in 1ml of saline with 1ml of Freund's complete adjuvant (Bacto, Difco Laboratories, Detroit, MI, USA), subcutaneously. After 10 days the rabbits were boosted with the same amount of antigen in Freund's incomplete adjuvant and bled from the ear 2 weeks after boosting. ELISA assays were performed in order to determine the presence of the desired antibodies in each serum.

ELISA

ELISAs were performed as described by McLaren *et al.* (1980).

SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Johnstone & Thorpe (1985). Samples were run in 10% polyacrylamide under reducing conditions. Gels blotted were run on a BRL model V16-2 apparatus (Bethesda Research Laboratories, Inc, Gaitherburg, MD, EUA).

Western Blotting

Western Blots were performed as described by Johnstone & Thorpe (1985). Transfer of proteins was checked by staining the nitrocellulose membrane either with 0.1% amido-black in 45% methanol and 10% acetic acid or by 1% Ponceau stain in 10% acetic acid.

Antibody Overlays

Antibody overlays were performed as described by Johnstone & Thorpe (1985). Nitrocellulose membranes were blocked with 0.3% Tween 20 in phosphate buffered saline (PBS) for 2 hours and then incubated with antisera diluted 1:200 in PBS containing 0.05% Tween 20 for 90 minutes at $37^{o}C$. After extensive wash, membranes were incubated with 8 x 10^{6} cpm of [125I]-protein A in PBS Tween 0.05% containing 3% albumin, at $37^{o}C$ for one hour. The membrane was extensively washed, air dryed and autoradiographed. Alternatively, membranes were incubated with goat anti-rabbit IgG conjugated with peroxidase (SAPU, Scottish Antibody Production Unit), and stained with diamino benzidine and α-chloronafthol.

Periodic Acid Treatment

Nitrocellulose membranes and ELISA plates were treated with periodic acid sodium borohydride as described by Woodward *et al.* (1985). The concentration of periodic acid used was 20mM.

Lectin overlays

Concanavalin A-peroxidase overlays were performed as described by Bouvier *et al.* (1985).

Ultraviolet irradiation

(1) The source:

A high-intensity ultraviolet lamp (Mineralight and Blak Ray model UVG-58, from UVP Limited), delivering its peak utput at 254nm, was used as the source of UV Radiation. The UV bulb in this lamp is in the form of a thin tubular strip.

The lamp was stationed in the horizontal position with metal clamps, and its output at 254nm measured using a UVX digital radiometer with a 254nm UV sensor (both from UVP Limited). A warm-up time of approximately 10 minutes was necessary to ensure a stable intensity of output. A platform was set up at 10cms below the lamp. At this distance, the radiation intensity varied both along the length of the UV bulb, and with distance to either side of the thin UV tube. The position was marked where radiation output was at a maximum. UV intensity at this point was 250μw cm^{-2}, varying by approximately $\pm$ 7.0% within a 3cm radius.

(2) Irradiation procedure:

The UV lamp was allowed to warm up. Its output was routinely checked, usually at the beginning of each experiment.

Cercariae collected after a 2-hour period of shedding were counted and used at a concentration of 500-800/ml. If necessary, they were concentrated on a Sinter glass filter (model P40; Gallenkamp), shining a beam of light onto the cercarial suspension to prevent the parasites sinking to the base of the filter. 8ml of the cercarial suspension were then pipetted into a sterile plastic Petri dish of 6cm diameter, 1.5cm depth (Becton, Dickinson Labware), and carefully placed at the optimal position below the UV lamp. Irradiation was carried out for the required time, usually 90 seconds, supplying 350-400μw min cm^{-2} (ie. 21-24 Joules per cm^2) of ultraviolet energy. This radiation dose was chosen in accordance with the work of Dean *et al.* (1983), who showed that *S. mansoni* cercariae exposed to UV irradiation at 330-440μw min cm^{-2} were fully attenuated, and induced optimal immunity to reinfection in NIH mice.

The irradiated cercariae were dispersed into test-tubes or universals as required for individual experiments. Any cercariae adhering to the bottom of the Petri dish were washed out by adding a further 2mls of aquarium water, and agitating gently with a pipette. A fresh Petri dish was used for each batch of cercariae to be irradiated.

Periodate-treatment and addition of competing sugars

Sodium meta-periodate was obtained from Sigma. The procedure for periodate treatment of schistosomula was adapted from the methods described by Omer-ali *et al.* (1986).

Schistosomula were formaldehyde-fixed (0.1% formaldehyde, 15 min), and washed throughly before treatment. Each sample of schistosomula was

then divided in two. One lot was treated with 10mls of a freshly made-up
solution of 0.05M sodium periodate in 0.05M sodium acetate, pH 5.8. The
parasites were incubated for 1 hour in the dark at room temperature. The
reaction was stopped by adding glycerol (stock solution made up in PBS) to
give a final concentration of 0.1M. After incubation in the presence of
glycerol in the dark for 20 minutes, the schistosomula were pelleted by
centrifugation, and washed 5 times in ice-cold GMEM.

In some experiments, immunofluorescence proceeded immediately after
periodate treatment and washing. In others, both the periodate-treated and
untreated samples were divided into two aliquots of 50μl, each containing
approximately equal numbers of schistosomula. To one member of each pair,
100μl of GMEM containing methyl(α-D-mannopyranoside, D(+)-galactose and
N-Acetyl=D-glucosamine (all from Sigma), each at a concentration of 0.4M
was added. The other member of each pair received 100μl of GMEM only.
After mixing, 50μl of the appropriate mouse serum was added, made up in
GMEM to give a concentration of 1/10 in the final 200μl volume.

After incubation for 1 hour at 37°C, FITC-conjugated rabbit anti-
mouse IgG (whole molecule) was added at 1/40 dilution.

RESULTS

1. Variable expression of antigens on the cercarial surface

When antibodies to snail or glycocalyx preparations bind to
populations of cercariae, considerable variability is observed (Fig. 1).
Other surface properties show this variability (Jones, Helm and Kusel,
1989). During natural infection, individual cercariae might deposit very
different concentrations of a variety of antigens in tissue migration
sites.

2. The effects of irradiation

(a) Retention of glycocalyx

Western blotting of mechanical or skin schistosomula using a
variety of antisera (αG1, αG2, αSH) indicate that after UV irradiation
the cercarial glycocalyx is retained on the surface membrane for several
hours longer after transformation than unirradiated controls. This
suggests that during an infection with irradiated cercariae, the
glycocalyx may be deposited in tissue sites different from normal.

(b) Induction of anti-carbohydrate antibodies

During both natural infection and infection with irradiated
cercariae, antibodies against periodate sensitive and periodate resistant
epitopes are induced. It can be shown that both periodate sensitive and
resistant epitopes bind antibodies in sera from mice infected with either
normal or irradiated cercariae (Figs. 3,4). A mixture of sugars can
compete with antibody binding. These antibodies are thus likely to be
binding to carbohydrates, epitopes, even after periodate treatment. These
results show that antibodies from a normal infection bind with a higher
affinity to normal schistosomula than to irradiated schistosomula (Fig. 3).

Hence, irradiation causes some changes in the structure and immunogenicity of the surface carbohydrates.

(c) <u>Inhibition of glycoprotein synthesis</u>

Irradiation can be shown to inhibit both ^{3}H mannose and ^{35}S methonine incorporation into TCA precipitable macromolecules. It is unclear whether this inhibition affects the surface properties of the parasite for the first 24 hours, since during this time a considerable portion of membrane synthesis occurs from presynthesised vesicles. However, over the period during which irradiated schistosomula survive (6-10 days), modified proteins might be expected to be secreted and processed by the host's immune system.

(d) <u>Inhibition of glycoprotein synthesis by tunicamycin</u>

To examine in detail the effects on the surface of schistosomula of an inhibition of glycoprotein synthesis, the effects of tunicamycin (TM) was studied. Inhibition of ^{3}H mannose incorporation was seen during 24hr incubation with TM (10 g ml^{-1}). An increase was detectable in the binding of a variety of lectins to the surfaces of treated schistosomula. This unexpected effect may result from the incorporation of incompletely processed proteins into the membrane. No ultrastructural changes in the surfaces of TM treated schistosomula or adult worms could be observed.

Tunicamycin acts in a manner similar to irradiation in that glycoprotein synthesis is inhibited and the schistosomula do not develop beyond the lung stage. Inhibition of glycoprotein synthesis for a short period of time (24hr) is sufficient to prevent development, perhaps because of the absence in the membrane of a crucial glycoprotein receptor. We are currently investigating whether TM inhibited schistosomula can induce protective immunity in the same way as irradiated schistosomula.

3. <u>Biophysical changes in surfaces after TM treatment</u>

Fluorescence recovery after photobleaching (FRAP) studies revealed no alteration in lateral diffusion of either lipid or glycoprotein after TM treatment. FITC-dextran was partially immobilised by parasite surfaces in both normal and TM treated schistosomula and it has been suggested that extensive carbohydrate chains at the parasite surface might create a microenvironment which is important for parasite survival (Kusel and Gordon, 1989). The synthesis of a high molecular weight glycoprotein (GP 170) (MacGregor and Kusel, 1989) in the adult surface may be responsible for maintaining these surface carbohydrate chains.

DISCUSSION

The great variability in expression of surface proteins and glycoproteins between individual cercariae implies that each antigen presenting cell of the host may be exposed to a different concentration of a mixture of macromolecules. Some of these macromolecules cross-react with snail epitopes and snail haemolymph and soft tissue glycoproteins can

inhibit the proliferative response of PMN in culture, as has also been shown for glycocalyx fractions (Vieira, Gazzinelli, Kusel, De Souza and Colley, 1986). Thus, proliferative responses of tissue T cells during migration of individual parasites are likely to be highly variable in the host.

A very different situation may exist when the host is infected with irradiated cercariae. Such cercariae may retain the glycocalyx for longer in the tissues, so that skin antigen presenting cells (APC) may be exposed to an altered proportion of inhibiting macromolecules. Furthermore, the irradiation inhibits protein and glycoprotein synthesis, so that some secreted molecules may be considerably modified, and processed more effectively by APC. The lack of heat shock proteins (HSP) normally induced during transformation might also lead to secretion or release of denatured proteins from irradiated forms. Glycoprotein synthesis soon after transformation is crucial for further growth, since inhibition by tunicamycin completely prevented parasite development, even though minimal disruption to the morphology or biophysical properties occurred.

The humoral response to irradiated schistosomula yields antibodies able to bind with high affinity to carbohydrate moieties on irradiated larvae, but with lower affinity to those normal schistosomula (Fig. 4). This demonstrates that antigens released by irradiated cercariae are processed by APC differently from those released by normal parasites. A summary of our view of the effects of inhibitors on glycoprotein synthesis is shown in Fig. 5. Further studies on the presentation of antigens from irradiated parasites might concentrate on the polypeptide and carbohydrate conformations of secreted glycoproteins.

SUMMARY

The cercarial glycocalyx and schistosomulum surface contains a number of glycoproteins which are expressed in very variable amounts within a parasite population.

Tunicamycin inhibits glycoprotein synthesis of schistosomula if the parasites are incubated for 24hr with the drug ($10\mu g$ ml^{-1}). An unexpected increase in lectin binding to the parasite surface was observed but no other changes were detected. Schistosomula treated in this way did not develop in the host past the lung stage. Ultraviolet irradiation ($400\mu W$ min cm^{-2}) also inhibited glycoprotein synthesis. Synthesis of other proteins, and in particular heat shock proteins, were also inhibited. Sera from mice (NIH strain) infected with irradiated cercariae contained antibodies which bound to normal schistosomula with lower affinity than to irradiated parasites. This is evidence that irradiation modifies the surface and secreted glycoproteins of schistosomula, so they are processed in a different way to normal glycoproteins by the host's immune system. The effects of irradiation on heat shock protein synthesis may allow the parasite to release a variety of proteins and glycoproteins in abnormal conformations. This may explain the enhanced immunogenicity of irradiated cercariae.

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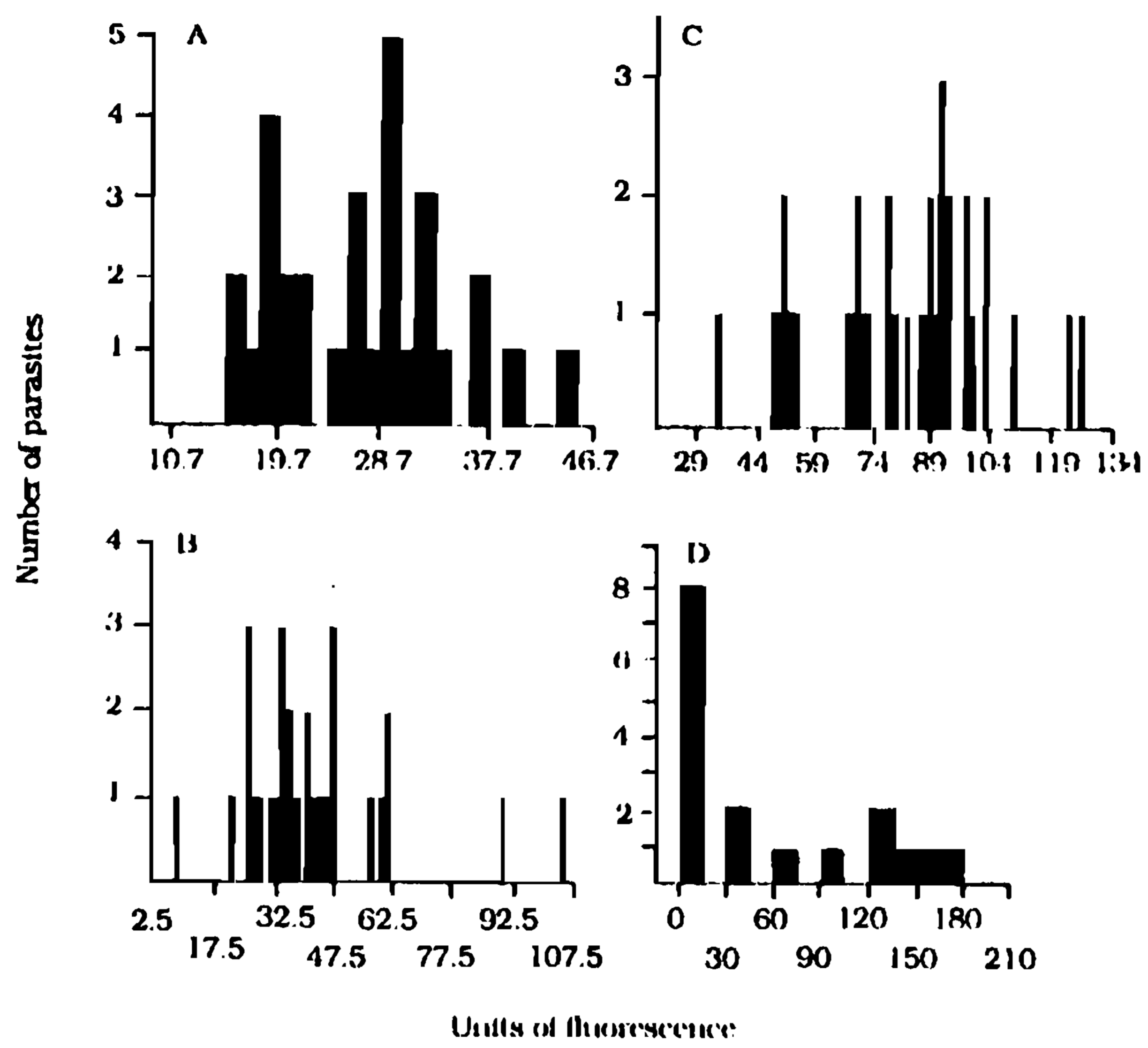Woodward M.P., Young W.W. & Bloodgood R.A. (1985)   J. Immunol. Meth, 78, 143-153.

Fig. 1 - Histograms showing the quantitative binding of rabbit anti-G1 (glycocalyx) antiserum to cercariae. A mixed population B.C.D clones of cercariae.
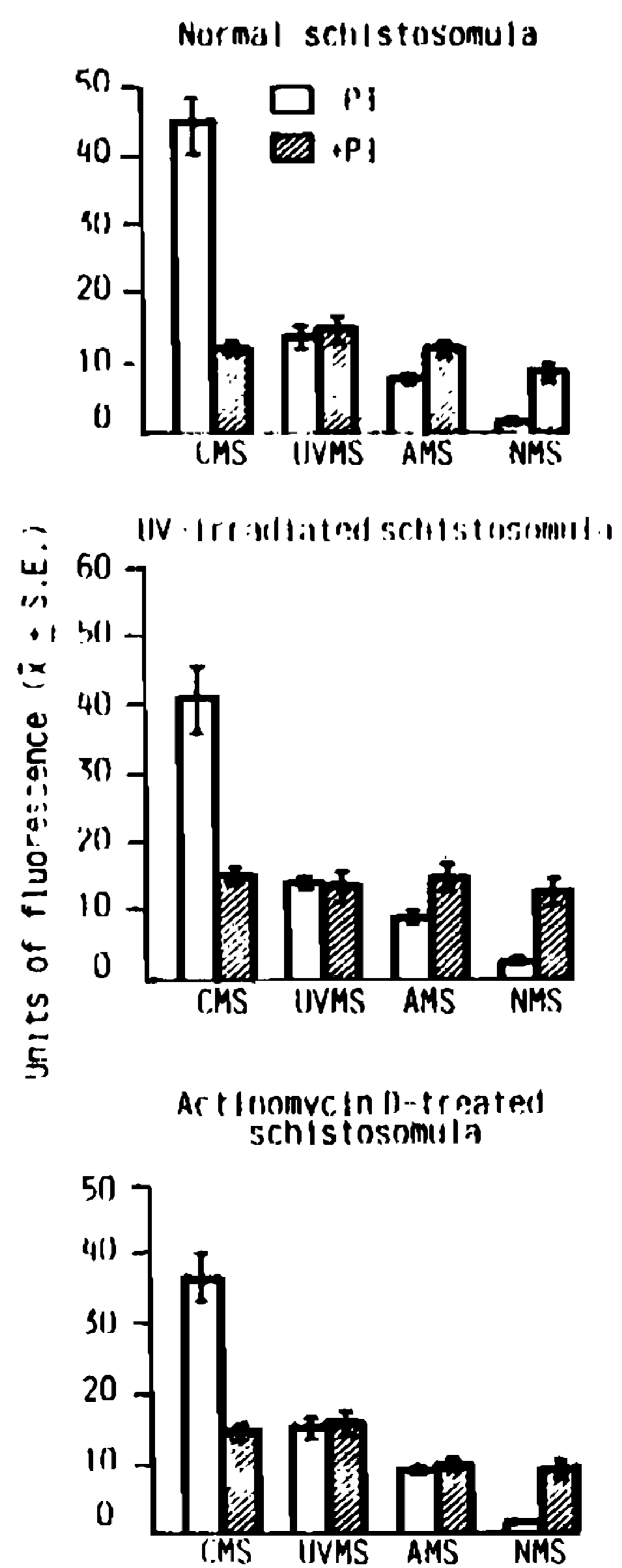

Fig. 2 - Binding to schistosomula of sera from mice infected with (a) normal cercariae (CMS), (b) UV irradiated cercariae (UVMS), (c) Actinomycin D treated schistosomula (AMS). NMS represents normal mouse serum. +PI represents periodate treated schistosomula.
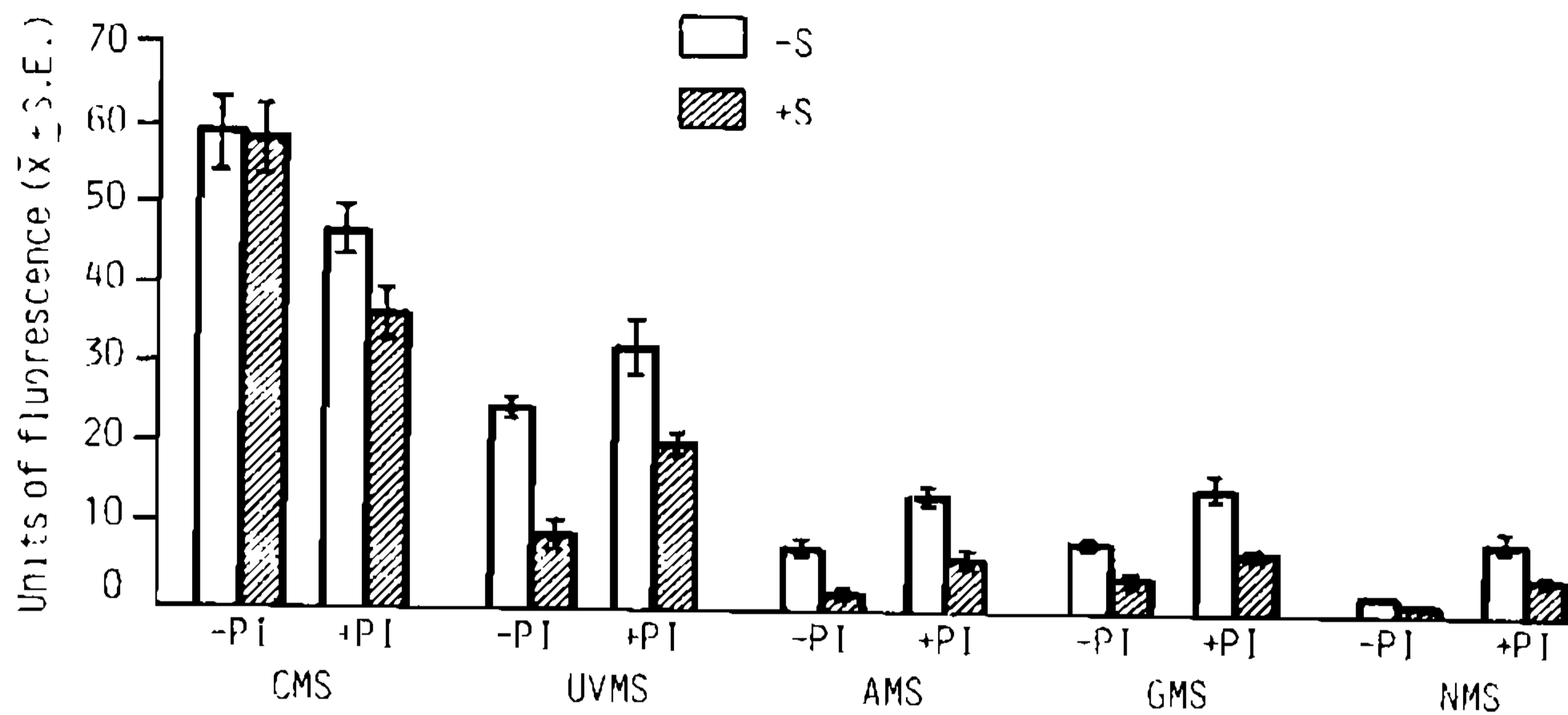
Fig. 3 - Binding to normal schistosomula of sera from normal cercariae
(CMS), UV irradiated cercariae (UVMS), Actinomycin D treated
schistosomula (AMS) and gamma irradiated cercariae (GMS). NMS is
normal mouse serum. +PI represents periodate treated
schistosomula. +S represents the presence of a competing sugar
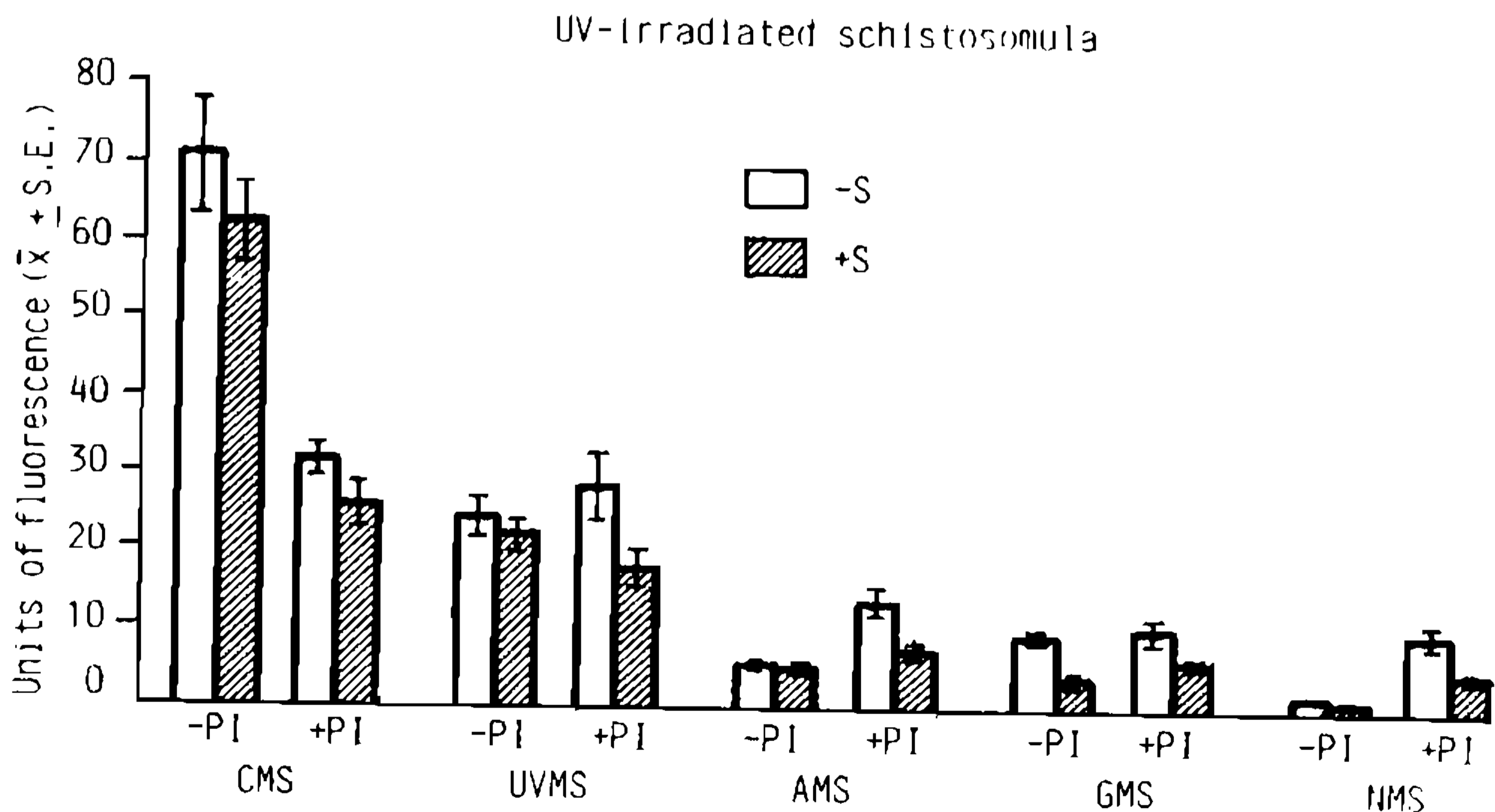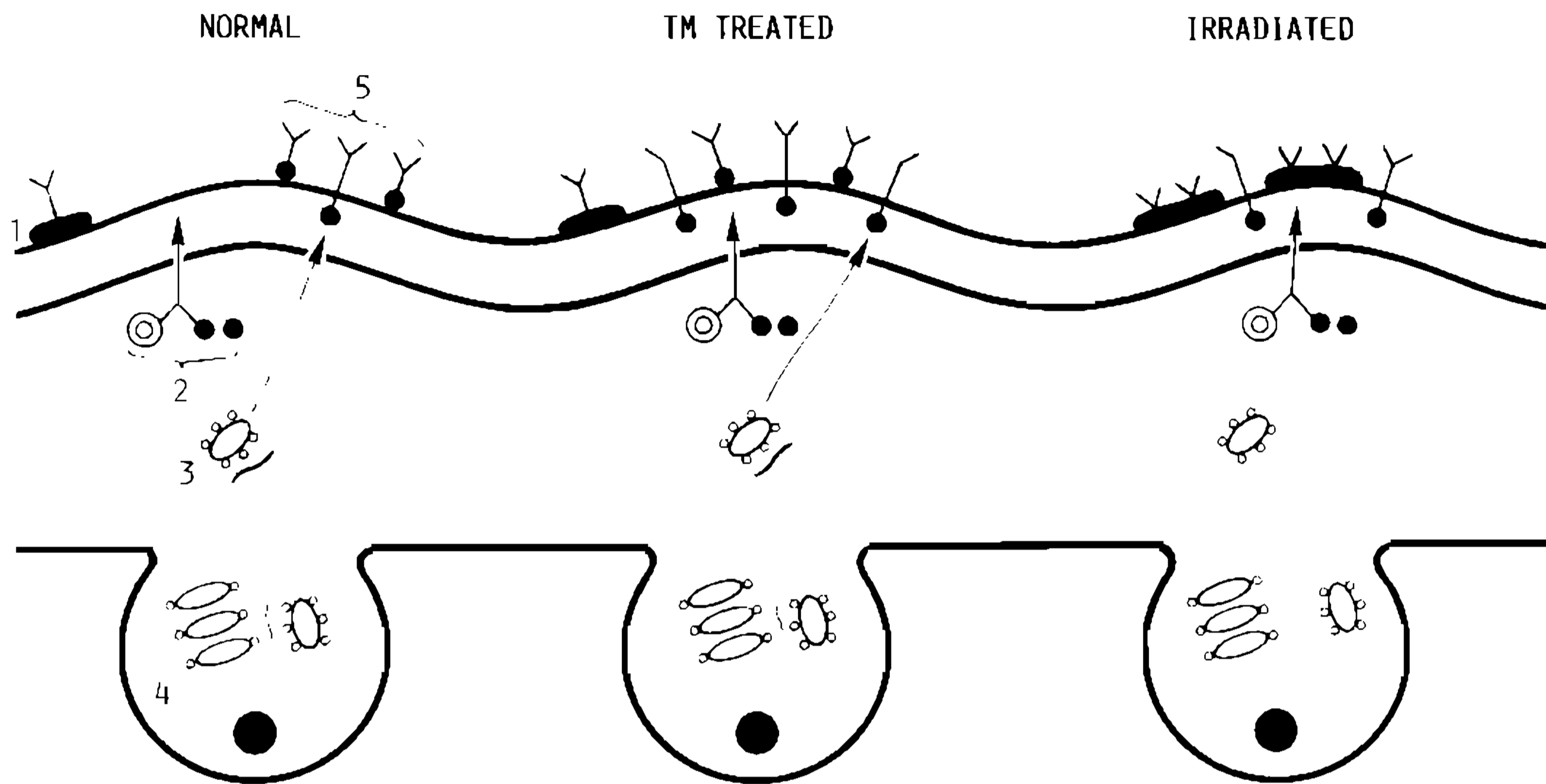mixture.

Fig. 4 - As in figure 3 except binding of sera is to UV-irradiated
schistosomula.

NORMAL	TM TREATED	IRRADIATED

NORMAL	TM TREATED	IRRADIATED
1. Glycocalyx lost.	Glycocalyx lost.	Glycocalyx retained alterations in carbohydrate and protein structure.
2. Presynthesised glyco proteins incorporated.	Normal	Normal
3. mRNA active.	Active	Inhibited.
4. N-linked glycosylation	Inhibited partially.	Inhibited partially.
5. Exposed glycoproteins.	Modified in newly synthesised proteins.	Modified by direct irradiation effects, and in newly synthesised proteins.