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Resistance induced by drug abbreviated *Schistosoma mansoni* infections: treatment with the drug Ro11-3128 leads to enhanced antigen presentation

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SUMMARY

Treatment of mice with the benzodiazepine derivative Ro11-3128 1–2 days post-infection with *Schistosoma mansoni* leads to arrest of virtually all schistosomula at the skin stage, and results in the development of protective immunity to challenge infection. A characteristic feature of Ro11-3128 treatment *in vitro* is the formation of exudates and membranous blebs at the schistosomular surface; other drugs tested, such as Ro15-5458 and oxamniquine which are also effective against the skin stages but relatively ineffective in inducing protection, do not induce this reaction. Here, we have examined whether such *in vitro* treatment causes enhanced presentation of schistosomular antigens by host antigen-presenting cells (APC) using an *in vitro* assay with activated peritoneal adherent cells as APC and T cells from *S. mansoni* antigen-sensitized mice. We have shown that viable mechanically transformed schistosomula (MS) can be processed and presented with similar kinetics to soluble antigen. However, *in vitro* drug treatment leads to enhanced presentation of MS. Experiments in which membranous blebs and antigen released by Ro11-3128-treated parasites during *in vitro* culture were separated from the remaining intact schistosomula, demonstrated significant stimulatory activity in the soluble and particulate-released antigen fractions. Filtration, antigen transfer experiments and SDS–PAGE analysis of the released material further suggested that most of the activity resided in the particulate fraction. Thus, quantitative and qualitative changes to antigen presentation by Ro11-3128 treatment early after infection may underlie the immunoprotective efficacy of Ro11-3128-abbreviated infections.

INTRODUCTION

Significant resistance to *Schistosoma mansoni* cercarial challenge in mice can be induced by termination of infections at the skin stage of migration with the benzodiazepine derivative Ro11-3128.[1] This long-lasting and reproducible immunity to challenge infection is comparable to that induced by vaccination with radiation-attenuated cercariae. Oxamniquine and Ro15-5458, which also result in parasite attrition when given at the skin stage, result in significantly lower levels of resistance than treatment with Ro11-3128.[1,2] Uniquely amongst these drugs, Ro11-3128 induces surface blebbing of the parasite membrane and we have suggested that these blebs may in some way facilitate antigen presentation.[2]

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Abbreviations: MS, mechanically transformed schistosomula; SDD, serum-derived drug—serum obtained 2 hr after oral Ro11-3128 administration.

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A number of lines of evidence are consistent with the idea that T-helper cell type 1 (Th1) cell-mediated immune mechanisms, such as those involving the activation of macrophages, are the major effector pathway in such 'single-dose' attenuated vaccine schedules, e.g. the inability of P strain mice to develop immunity;[3] the requirement for $CD4^+$ T cells during parasite attrition;[4,5] the cytokine profile, characterized by the production of interferon-γ (IFN-γ) but not interleukin-5 (IL-5);[6,7] the ablation of immunity by treatment with antibodies against INF-γ[8] or against IL-2 receptor.[9] Despite the fundamental role of T-cell mediated immunity in resistance to *S. mansoni*, the characteristics of antigen processing for this parasite, or indeed for other helminths, have not been described. Since most antigen-processing events occur intracellularly,[10,11] helminths pose unique problems to the host due to their large size. As antigen-presenting cells (APC) are unable to phagocytose directly the whole parasite, they are reliant on their ability to acquire soluble excretory/secretory antigens, or antigens recovered from the parasite membrane. In this study we have determined some of the basic parameters of the presentation of schistosomula *in vitro* and demonstrate that their treatment with Ro11-3128 leads to an increase in the levels of antigen available for presentation. It is believed that these are the first such observations of the processing and presentation of

antigens from a complex multicellular helminth parasite, and the results suggest a mechanism of action for Ro11-3128-attenuated vaccination.

MATERIALS AND METHODS

Parasite and laboratory hosts

A Puerto Rican strain of *S. mansoni* was maintained in *Biomphalaria glabrata* snails from Puerto Rico and random-bred TO and TFN mice (Tuck Ltd, Battlebridge, U.K.). For experimental work, C57BL/10 ScSn mice (NIMR, Mill Hill, U.K.) were used between 6 and 10 weeks old and weighing 20–25g.

Production and drug treatment of cercariae

Cercariae were shed in a minimum volume of dechlorinated water at 28° under direct illumination for 2 hr. The cercariae were concentrated, to a final volume of 10 ml, using Millipore concentration apparatus (Millipore Corp., Bedford, MA) fitted with an 8-μm filter (Schleicher and Schuell, Dassel, Germany).[12] Where sterile parasites were required for tissue culture, snails were washed several times in sterile water before shedding, and snail faeces were allowed to settle before decanting the cercarial suspension.

Production of mechanically transformed schistosomula (MS)

MS were produced as described elsewhere.[13] Earle's lactalbumin hydrolysate medium (ELAC; Flow Ltd, Ayrshire, U.K.) was buffered with 20 mM HEPES (N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid) and contained 0·85 g/l sodium bicarbonate, 25 μg/ml gentamycin (Sigma, Poole, U.K.), 100 U/ml penicillin (Glaxo Laboratories, Greenford, U.K.) and 100 μg/ml streptomycin (Evans Medical, Horsham, U.K.).

Heads and tails were separated by 10–12 passages through a 21-gauge needle. Tails were removed by sedimentation for 5 min before removing the supernatant. The heads were resuspended and incubated in ELAC at 37° for 3 hr. For sterile culture, parasites were sedimented five times in 20 ml ELAC medium under sterile conditions before incubation.

For storage, MS were pelleted by centrifugation and snap-frozen in liquid nitrogen and stored at −80°.

Drug treatment of parasites for use in antigen presentation assays

Serum-derived drug (SDD) was prepared by giving mice curative doses (175 mg/kg) of Ro11-3128, a benzodiazepine derivative (generously provided by Dr H. Stohler, Hoffmann-La Roche, Basel, Switzerland) as previously described. After 2 hr mice were bled, the blood allowed to clot overnight at 4°, and the clot removed. Serum was spun and diluted to 50% in RPMI before sterile filtration using a 0·22-μm filter (Schleicher and Schuell) and heat-inactivation for 30 min at 56°.

MS were incubated in the presence of 50% SDD, for 2 hr. Parasites were gently washed to remove the drug and/or residual serum by sedimentation through three changes of RPMI. Schistosomula were then incubated, at a concentration of 6000/ml, overnight in fresh RPMI-1640 medium (Gibco BRL, Paisley, U.K.), pH 7·2, containing the antibiotics described above and 2 mM glutamine (Gibco BRL), at 37°, 5% CO_2 in a humidified incubator before use. Unless otherwise stated, parasites were not washed after this overnight incubation. Viability, as judged by continual motility, was >80% after this overnight incubation.

Preparation of soluble antigen from MTS

One and a half million MS were combined in 2 ml cold sterile phosphate-buffered saline (PBS; 0·15 M sodium chloride, 2·68 mM potassium chloride, 8 mM disodium hydrogen orthophosphate, 1·47 mM potassium dihydrogen orthophosphate) containing 1 μM phenyl methyl sulphonylfloride (PMSF; Sigma) on ice. These were homogenized, sonicated and spun for 1 hr at 100,000 g. The protein concentration of the supernatant was estimated using the Biorad assay using bovine serum albumin (BSA) as a standard (Biorad, Hemel Hempstead, U.K.). Aliquots were adjusted to 1 mg/ml with PBS, filter sterilized using a 0·22-μm filter, and stored at −70°.

Cell culture media

For cell culture, complete medium (RP) consisted of HEPES-buffered Dutch modified RPMI-1640 medium (Gibco BRL) pH 7·2, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 0·05 mM 2-mercaptoethanol and 10 mM sodium pyruvate (Gibco BRL). Heat-inactivated fetal calf serum (FCS; low endotoxin; Gibco BRL) was added at the indicated concentrations (e.g. RP5, 5%; RP10, 10%). Cells were maintained in a humidified incubator at 37° with 5% CO_2.

Preparation of cell populations

Single-cell suspensions were obtained, from caudal lymph nodes, using a sterile 125-micron aperture nylon filter mesh sieve (Cadisch and Son, London, U.K.). Red blood cells were lysed by addition of 1·7 mM Tris-buffered 0·83% ammonium chloride, pH 9·6 for 10 min at 37°. Cell suspensions were washed three times in RP1 by centrifugation for 10 min at 1000 *g*. Viable cell counts were made in 0·2% trypan blue (Sigma) in 0·15 M PBS, pH 7·2.

Isolation of T cells from lymph nodes

Schistosoma mansoni immune T cells were prepared from the draining lymph nodes of mice immunized subcutaneously (s.c.), 6–7 days previously, with homogenized, sonicated MS emulsified in complete Freund's adjuvant (CFA) (Difco Labs, Detroit, MI). T cells were separated from lymph nodes by nylon wool filtration.[14,15] Briefly, 10^8 lymph node cells were loaded onto sterile 600-mg nylon wool columns in 1 ml RP20. After incubation at 37° for 45 min, cells were eluted with RP20 at 37°. The cells were then passed through fresh columns (as above) followed by a 2-hr plastic adherence step in RP10, before counting and plating for assay. As determined by flow cytometric analysis, over 85% cells were positive for Thy-1 antigen expression (data not shown).

Isolation of peritoneal APC

Peritoneal exudate cells, from mice given an intraperitoneal injection of 200 μg concanavalin A (Con A) in 0·5 ml saline 5 days previously, were used as a source of APC. Peritoneal lavage was performed with 5 ml cold RPMI and cells were washed twice in polypropylene tubes. These were plated at 10^5/well in Nunc FB96 well plates (Roskilde, Denmark) in RP10 and allowed to adhere for 2 hr at 37°. Non-adherent cells were removed by washing. The adherent population was 50–70% class II-positive, as determined by immunofluorescence using monoclonal antibody (mAb) M5/114 (data not shown).

Antigen-presentation assays

Adherent peritoneal cells were washed twice with serum-free medium before incubation with soluble antigen or with intact or variously treated MS diluted in RP10. To terminate antigen processing, cells were washed with RPMI and fixed with 1% paraformaldehyde (Sigma) in PBS, pH 7·2, for 15 min, followed by quenching with 0·1 M lysine (Sigma) in PBS for 15 min. After two washes in serum-free medium, the monolayers were maintained overnight or for a minimum of 4 hr, at 37° in RP10, before the addition of the purified T-cell population.

T cells, $1-2 \times 10^5$/well, were added in a final volume of 200 μl RP5 medium supplemented with 5×10^{-5} M 2-mercaptoethanol. After 4 days, 100 μl supernatant was removed and stored at −20° for cytokine assay. Cultures were then pulsed with 0·5 μCi (18·5 kBq) [^{3}H]thymidine ([^{3}H]TdR; specific activity 5 Ci/mMol; Amersham Int., Amersham, U.K.) for 6 hr before harvesting. Incorporation of [^{3}H]TdR was determined by scintillation counting and results are expressed as the mean d.p.m. for triplicate cultures ± SE Statistical significance was assessed using the Student's *t*-test.

Functional assessment of IL-3/GM-CSF production

The IL-3/granulocyte–macrophage colony-stimulating factor (GM-CSF)-dependent mouse cell line, FDCP-2, was maintained in RP10, supplemented with either 8–15% WEHI-3b supernatant or a 1/100 dilution of murine recombinant IL-3 (mIL-3) in 5% CO_2 at 37°. Original stocks of FDCP-2 cells were the kind gift of Dr Dexter (Patterson Labs, Christie Hospital, Manchester, U.K.). WEHI-3b, an IL-3-producing mouse myelo monocytic cell line[17] was maintained in RPMI supplemented with 5% horse serum (Sigma), and the recombinant murine IL-3 was produced by transfected X63Ag8 myeloma cells.[18]

Cells were prewashed and plated in flat-bottomed 96-well plates at 10^5 cells/well in RPMI, 5% FCS (RP5). Test supernatants were added in triplicate wells to a final concentration of 20% in 100 μl, and cells incubated for 72 hr. FDCP-2 proliferation was determined using the chromogenic salt MTT (3-(4-5-dimethyl thiazol-2-yl) bromide; Sigma), as described elsewhere.[19]

SDS–PAGE analysis of proteins

To analyse released schistosomular proteins in the absence of serum components, larvae were treated with Ro11-3128 dissolved directly in culture medium rather than in the form of SDD. A dilution of drug was chosen which had a similar effect on schistosomula survival to the SDD described above, i.e. greater than 80% of larvae were viable after 24 hr.

Proteins were separated on 12% SDS–PAGE gels[20] and visualized by silver staining.

RESULTS

Activated peritoneal APC present antigen to T cells previously primed with *S. mansoni* antigen *in vivo*

To address the specific question as to whether drug-treated parasites are more effectively presented than non-drug-treated parasites and to minimize other variables, we chose to use an assay involving Con A-activated peritoneal APC and T cells

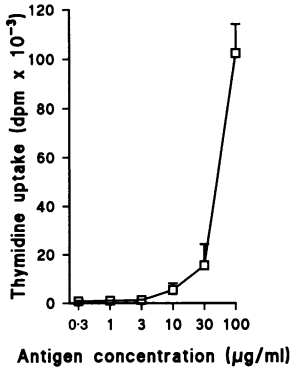

Figure 1. Presentation of soluble MS antigen by fixed APC. Con A-activated peritoneal adherent cells were pulsed for 3 hr with varying concentrations of MS antigen, washed and fixed in paraformaldehyde. Thymidine incorporation by added *S. mansoni*-specific T cells was determined 4 days later. Data are expressed as the mean ± SE of triplicate cultures.

obtained by priming with a single batch of MS antigen. Use of the same batch of injected antigen throughout these experiments provided a reproducible priming and read-out system.

Prior to examining the presentation of whole schistosomula, the conditions of the antigen presentation assay were optimized using soluble MS antigen. Con A-activated peritoneal APC were pulsed for 3 hr with soluble MS antigen before washing, fixation and addition of antigen-primed lymph node T cells. As shown in Fig. 1, there was a dose-dependent T-cell response to soluble MS antigen under these conditions, reaching a maximum at 100 μg/ml (102,000 d.p.m. ± 11,800). In contrast, APC which had been fixed prior to incubation with antigen did not stimulate a T-cell response (910 d.p.m. ± 105) illustrating that an antigen-processing step was required for T-cell recognition. In the absence of the APC, purified T cells incubated with antigen over the entire 4-day assay period, showed negligible proliferation (< 2000 d.p.m.), confirming a lack of contaminating APC in these T-cell preparations.

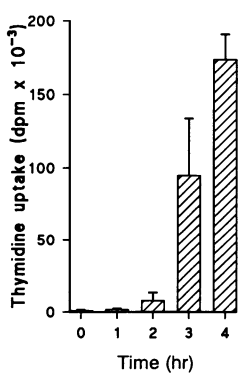

Figure 2. Kinetics of antigen processing of soluble MS antigen. Peritoneal APC were pulsed for the indicated times with 100 μg/ml MS antigen followed by fixation. Specific T cells were added and proliferation determined at day 4. Data are mean ± SE of triplicate cultures.

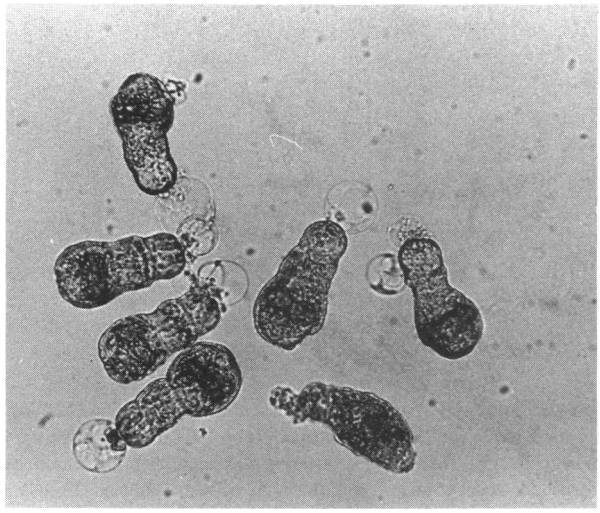

Figure 3. MS after Ro11-3128 treatment (50% SDD for 2 hr) followed by overnight culture.

To determine the kinetics of processing of soluble schistosomula antigen, APC were pulsed for varying lengths of time with 100 μg/ml soluble MS antigen before washing and fixation. As shown in Fig. 2, a processing period of at least 3 hr was required for significant T-cell recognition. Extending the processing period to 8 hr did not further increase the response (data not shown).

Ro11-3128-treated schistosomula stimulate higher T-cell responses than untreated schistosomula

MS were treated with Ro11-3128 by culturing for 2 hr in the presence of Ro11-3128 in the form of SDD (SDD-MS). After this period, over 90% of the schistosomula had one or more blebs but remained motile. SDD-MS were gently washed to remove drug without dislodging the blebs. Following overnight incubation, over 80% of the parasites were still alive, as judged by continuing motility (Fig. 3). Viability and percentage survival were comparable with MS cultured in medium alone

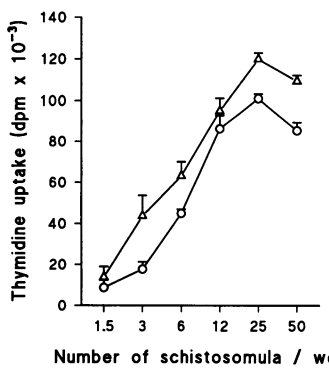

Figure 4. Dose–response curve of proliferation to MS or SDD-MS. Varying quantities of overnight cultures containing a known concentration of MS (○) or SDD-MS (△) were added to peritoneal APC, which were then used to present antigen as described in Fig. 1. The estimated number of MS or SDD-MS in each well is indicated.

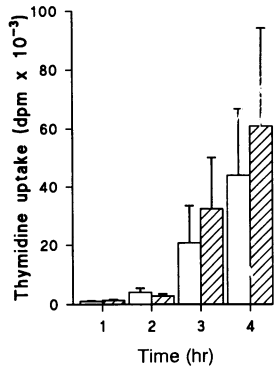

Figure 5. Kinetics of antigen processing of intact viable schistosomula. Whole viable MS (□) or SDD-MS (▨) (25/well) were added to peritoneal APC for the indicated times. APC were then fixed and sensitized T cells added. Data based on triplicate cultures were derived as described in Fig. 1.

and with MS cultured in normal mouse serum (NMS) (NMS-MS). Parasites were gently resuspended in the overnight culture medium and diluted appropriately for addition to the APC. In preliminary experiments, control MS were cultured in exactly the same way but in the absence of SDD. The data in Fig. 4 illustrates a representative experiment (of four) in which MS or SDD-MS were titrated onto APC and processing allowed to continue for 4 hr. As shown, exposure to these viable schistosomula efficiently triggered T-cell proliferation while in control experiments lacking APC such proliferation did not occur (< 2000 d.p.m.). It was observed, in this and in comparable experiments, that SDD-MS consistently stimulated higher responses than MS.

In subsequent experiments, to control for the presence of serum in SDD, control schistosomula were cultured in the same way as SDD-MS but with NMS in place of SDD (NMS-MS). The kinetics of the response to 25 NMS-MS or 25 SDD-MS were examined in three experiments, the results of one of which is shown in Fig. 5. APC were incubated for 1–4 hr with the schistosomula before fixation. The kinetics of presentation were similar to that seen for the soluble antigen in Fig. 1 and again SDD-MS produced greater proliferation than the non-drug treated schistosomula (NMS-MS).

In spite of some difficulties in accurately dispensing small numbers of worms the above series of experiments consistently showed that exposure of schistosomula to Ro11-3128 results in enhanced availability of antigen to APC.

Ro11-3128-treated schistosomula release high levels of antigenic material

In the above experiments, intact living schistosomula were cultured overnight, following exposure to Ro11-3128, and used without subsequent washing. Altered antigen presentation by Ro11-3128 treatment could therefore reflect either: (1) enhanced accessibility of whole MS-associated antigens to APC; (2) enhanced release of soluble and particulate (membraneous) material into the overnight culture medium; or (3) a combination of both. In order to establish which of these components was the prime source of the enhanced Ro11-3128-induced antigenic stimulus, the blebs were first separated

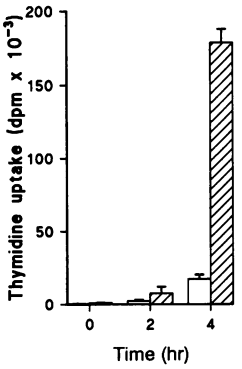

Figure 6. Presentation of bleb/released antigen derived from drug-treated MS. The bleb/released antigen fractions recovered (see Results) from MS treated either with NMS (□) or SDD (□) were added to APC at a concentration equivalent to that of 300 MS/well. After 2 or 4 hr processing time, APC were fixed and T cells added. Data, based on triplicate cultures, were derived as described in Fig. 1.

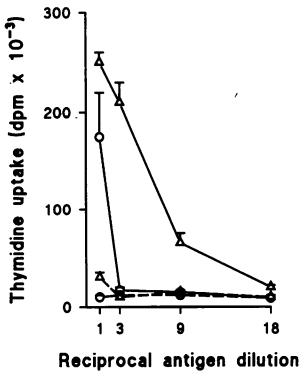

Figure 7. The stimulatory bleb/released antigenic material was depleted by incubation with APC. Varying dilutions of antigen derived from MS (○) or SDD-MS (△) were added to primary APC cultures for 4 hr (solid line). The antigen-containing supernatants were then transferred to secondary APC cultures for a further 4 hr (broken line). T cells were added to both sets of APC and proliferation assessed as described in Fig. 2.

from the parasite bodies by vigorous pipetting, microscopic examination confirming that most blebs had become detached. The intact parasites were then sedimented out, leaving a supernatant (SN) containing any soluble released antigen, together with shed particulate antigen including blebs. The concentration of this SN applied to APC was standardized with respect to the number of schistosomula incubated. As shown in Fig. 6, the SN from SDD-MS was highly immunogenic and stimulated approximately 10-fold greater T-cell responses than that obtained from an equivalent number of NMS-MS. Presentation of this antigenic material followed similar kinetics to both soluble antigen and total whole schistosomula populations. In addition to presentation of this material, isolated washed schistosomula, free of the soluble material released during overnight culture and largely free of blebs, were also able to be presented by these APC. At a concentration of 10 MS/well this was again somewhat higher for SDD-MS compared to NMS-MS (15,634 ± 6504 d.p.m.; 8835 ± 823 d.p.m., respectively).

Antigens are associated with blebs and particulate material in SN derived from Ro11-3128-treated schistosomula

In order to determine the availability of antigen in the mixed bleb/soluble antigen mixture, macrophages were incubated with a titration of SN derived from NMS-MS and SDD-MS. After 4 hr culture, SN were removed from the primary cultures and the pulsed APC fixed with paraformaldehyde. The SN was then added to fresh (secondary) APC cultures, also for a 4 hr pulse period, before fixation. As shown in Fig. 7 titration of the bleb/soluble antigen confirmed the previous observation that SN derived from SDD-MS contains more antigenic material than that derived from NMS-MS. Indeed, only at the highest concentration of NMS-MS antigen was proliferation observed. Strikingly, neither SN fraction was subsequently able to provide adequate antigen for processing when transferred to fresh secondary macrophage cultures.

One explanation for these data is that soluble antigens originally present in the SN were in limiting quantities and were thus exhausted in the primary presentation assay. Alternatively, the complete removal of antigenic material may reflect the efficiency of the APC in capturing particulate matter, especially the membranous blebs. Indeed, over a 30-min period, blebs were observed to settle out under gravity and thus may not have been recovered with the SN transferred from the primary to the secondary culture. This possibility, that blebs were primarily responsible for the stimulation observed, was investigated in the following experiment.

SN from SDD-MS was prepared as described above and divided into two samples. One of these was passed though a 0·22 μm filter and the resulting filtrate collected. Equal quantities of both filtered and unfiltered SN were then incubated with APC, which were washed and fixed after 4 hr. In the presence of APC previously incubated with the fraction containing blebs, T cells produced significant levels of response, measured in this experiment as IL-3/GM-CSF production (Fig. 8). Filtration of the supernatant was seen to almost totally remove its stimulatory activity.

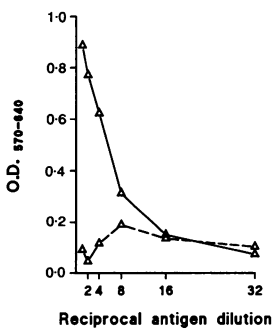

Figure 8. Filtration removed the stimulatory activity from SDD-MS bleb/released antigenic material. APC were pulsed for 4 hr with bleb/released antigen from SDD-MS which was either untreated (solid line) or passed through a 0·22 μm filter (broken line). T cells were added and the response determined by assaying day 4 culture supernatants for the presence of IL-3/GM-CSF (see the Materials and Methods).

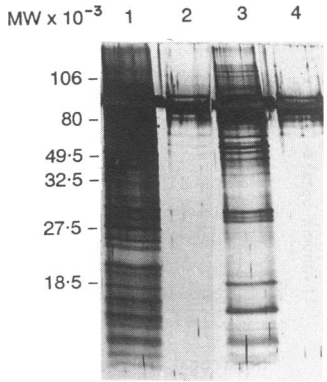

Figure 9. Protein composition of MS-derived antigenic material. SDS–PAGE analysis of unfiltered and filtered (0·22 μm) overnight culture medium from SDD-MS (lanes 1 and 2) and NMS-MS (lanes 3 and 4).

SDS–PAGE and analysis of proteins in culture SN derived from overnight incubation of NMS-MS and SDD-MS

To examine the protein composition of these fractions, samples were run on SDS–PAGE and silver stained. Fig. 9 shows a gel on which the unfractionated SN from SDD-MS and filtered SN was run. Each track contained the equivalent of protein produced overnight by 30 schistosomula. Filtration removed a large proportion of the protein content (lane 2) seen in the unfiltered fraction (lane 1) from SDD-MS. SN from schistosomula incubated with NMS contained a number of proteins, similarly removed upon filtration (lanes 3 and 4, respectively). This may explain the weak T-cell response seen on prior incubation of macrophages in some of these studies with NMS-MS (e.g. Fig. 6). Comparison of lanes 1 and 3, unfiltered SN from SDD-MS and NMS-MS, respectively, suggests that not only was there an increased antigen release in the drug-treated parasites but also that the SDD treatment caused qualitative differences in the proteins seen.

DISCUSSION

We have previously suggested that the efficacy of infections terminated at the skin stage with Ro11-3128 in inducing immunity in mice may be related to altered/enhanced antigen presentation. This could arise from the effect the drug is observed to have on the skin stage schistosomula, i.e. production of exudates and membranous vesicles (blebs) at the parasite surface.[9] The present experiments were designed to compare antigen presentation from normal or Ro11-3128-treated schistosomula *in vitro*. The results show that antigen from viable, non-drug-treated schistosomula of *S. mansoni* can be presented by APC *in vitro* but that the efficiency of presentation is markedly enhanced by pre-treatment of schistosomula with the drug Ro11-3128, a phenomenon associated with a particulate fraction of released antigen.

Antigens are known to be released from normal schistosomula both *in vitro* and *in vivo*. Membrane proteins labelled with iodine have been shown to be lost *in vivo* much more rapidly than during *in vitro* culture[21]. However, antigens are also lost from the larval surface during *in vitro* culture.[22] Thus during 24–48 hr in culture certain surface antigens of MS become unavailable for surface iodination and some, iodinated prior to culture, can be recovered from culture medium. Some surface antigens have been shown to be glycosyl-phosphatidylinositol (GPI)-anchored to the membrane,[23,24] which may account for the selective release of certain antigens. Molecules released from schistosomula *in vitro* have been shown to include antigens recognized by IgE from immune rats,[25] including a 26,000 MW antigen which is the target of a protective mAb.[26] In addition to the release from the schistosomula surface of membrane-attached antigens, certain enzymes with a normally cytosolic location have been reported to occur at the larval surface and have been implicated in protection, e.g. glutathione-S-transferase (GST),[27] triose phosphate isomerase (TPI)[28] and glyceraldehyde-3-phosphate dehydrogenase.[29] Their apparent lack of membrane-attachment structures, and, in the case of GST and TPI, their transient appearance at the larval surface, suggests that such antigens would be released during *in vitro* culture.

The demonstration of drug-induced production of membranous blebs at the parasite surface leads intuitively to the suggestion that this could enhance both the range and quantity of antigenic material released. We have previously demonstrated certain larval surface antigens on the blebs which do not appear to be derived from the existing outer leaflet of the larval surface membrane.[9] These blebs may also contain soluble cytosolic antigens. SDS–PAGE analysis presented here again demonstrates both quantitative as well as qualitative differences in antigen release due to drug treatment.

Although we cannot be sure that blebbing occurs *in vivo*, it is clear that such drug-terminated infections result in high levels of antibody against larval surface antigens.[9] The amount and range of such anti-surface antibody varies according to the larval stage targeted *in vivo*, being highest when treatment is directed against 1–4-day-old parasites and progressively less with 6- or 15-day-old infections. This parallels the levels of resistance induced *in vivo* and a decline in the production of membranous vesicles by progressively older stages. In addition, other larvicidal drugs studied by us, e.g. Ro15-5458, which also kills larvae at the skin stage, are markedly less effective than Ro11-3128 in stimulating immunity and these do not cause blebbing *in vitro*.[1,9] Altered antigen release and/or more effective presentation of antigens of skin stage larvae may thus be pivotal to the development of optimal immunity. Although in the present *in vitro* studies the enhanced proliferation was of the order of two to three times greater with Ro11-3128-treated MS than with controls, in the effective protocols used *in vivo* many more parasites are used and Ro11-3128 would have a protracted influence on the larvae, which have been shown to survive for several days. Within the skin, Langerhans' cells are the resident APC, with later support from inflammatory monocytes. Although these APC have characteristics distinct from peritoneal APC, they are both able to effectively handle particulate and soluble antigens.[11] Indeed, Langerhans' cells are probably required for antigen transfer to the draining lymph node (by cellular migration) and for initiation of the primary T-cell response at that site. This stage in immunity would be significantly enhanced by greater antigen availability. Furthermore, a proportion of the larvae reach the lymph nodes, a feature of Ro11-3128 attenuation which may be important in induction of immunity.[30] Thus protracted and enhanced antigen release, perhaps also

occurring within lymph nodes may together contribute to the efficacy of Ro11-3128-terminated infections.

Antigens derived from non-drug-treated living schistosomula were shown to be functionally available for T-cell recognition within 3–4 hr of the interaction of antigen with APC. This was somewhat longer than the 1–2 hr for *Cryptococcus neoformans*[31] but similar to that seen for *Leishmania donovani*,[32] and may reflect either the path of processing through the APC, the availability of antigen to the APC, or the ease of processing of immunodominant epitopes. Despite the apparently different forms of antigen studied here, the similarity in kinetics of processing probably reflects the fact that antigen acquisition is not a limiting factor.

When treated with Ro11-3128 in a form comparable to that which would be experienced by schistosomula in the skin of mice given a curative dose, i.e. 50% SDD, MS subsequently showed enhanced release of antigenic material compared with control MS. The schistosomula were only exposed to SDD for 2 hr and the released antigen subsequently collected during a further 14–16 hr in culture. Even after this overnight culture and washing to remove blebs, drug-treated schistosomula remained viable. Similarly, following the transient exposure to Ro11-3128 which occurs during *in vivo* treatment, significant numbers of schistosomula have also been shown to survive for several days.[30] It was found to be possible to remove a large proportion of the stimulatory antigen by filtration, indicating that the drug-induced membranous blebs or associated antigens play a significant role in stimulation of previously primed T cells. SDS–PAGE analysis of the filtered and non-filtered supernatants showed that the majority of proteinaceous material was associated with the particulate fraction. This was consistent with the failure to transfer stimulatory activity to fresh macrophages with the antigen-containing supernatants recovered from primary cultures after 4 hr. Here it was found that the majority of blebs remained associated with the primary cultures, and this material settled out under gravity in under 30 min. The possible exhaustion of antigenic activity in the soluble fraction of the transferred supernatant was considered unlikely given that in other studies on the uptake of soluble antigens only approximately 0·01% of available antigen is taken up by fluid-phase uptake during a 1 hr pulse period.[33] Others have also shown membranous blebs to contain immunogenic material, capable of stimulating human peripheral blood lymphocytes.[34]

Although it has been demonstrated here that *in vitro* drug treatment of schistosomula with Ro11-3128 increases release of a particulate/membranous antigenic fraction, it remains to be established whether this includes the protective antigens responsible for immunity in this model. Questions also remain as to whether priming of a comparable T-cell population occurs during protective Ro11-3128-attenuated vaccination *in vivo*.

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