enhance senescent IgG binding by more than three orders of magnitude. Therefore, expression of the senescence marker requires neither covalent modification of band 3, nor exposure of a cryptic antigenic site as previously suggested; the clustering of a few copies of band 3 results in a substantial increase in IgG binding to senescent cells. We have shown that hemoglobin denaturation is one mechanism by which this clustering can occur. The near absence of Heinz bodies in RBC from normal individuals and the abundance of Heinz bodies in RBC from normal individuals after splenectomy (that is, after a major site of senescent cell phagocytosis has been removed) indicates that this mechanism is operative in vivo (*12*). Immunofluorescence results from our laboratories demonstrate that the sites of Heinz body binding in untreated cells from splenectomized patients coincide with the sites of both band 3 clustering and autologous IgG binding (*22*).

PHILIP S. LOW*
STEPHEN M. WAUGH
Department of Chemistry,
Purdue University,
West Lafayette, Indiana 47907
KAREN ZINKE
DETLEV DRENCKHAHN
Institut für Anatomie und Zellbiologie,
Universität Marburg, D-3550 Marburg,
Federal Republic of Germany

References and Notes

1. M. M. B. Kay, K. Sorensen, P. Wong, P. Bolton, *Mol. Cell. Biochem.* **49**, 65 (1982); W. H. Crosby, *Blood* **14**, 399 (1959).
2. G. M. Shaw, D. Aminoff, S. P. Balcerzak, A. F. LoBuglio, *J. Immunol.* **125**, 501 (1980).
3. G. Bartosz, M. Soszynski, A. Wasilewski, *Mech. Ageing Dev.* **20**, 223 (1982).
4. M. M. B. Kay, *J. Supramol. Struct.* **9**, 555 (1978).
5. R. F. Todd III, R. A. Torchia, K. E. Peterson, E. L. Leeman, *Clin. Immunol. Immunopathol.* **30**, 413 (1984).
6. N. Khansari and H. H. Fudenberg, *Cell. Immunol.* **78**, 114 (1983).
7. H. U. Lutz and G. Stringaro-Wipf, *Biomed. Biochim. Acta* **42**, 117 (1983).
8. M. M. B. Kay, S. R. Goodman, K. Sorensen, C. F. Whitfield, P. Wong, L. Zaki, V. Rudloff, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1631 (1983).
9. H. U. Lutz, *Schweiz. Med. Wochenschr.* **111**, 1507 (1981).
10. S. M. Waugh and P. S. Low, *Biochemistry*, in press.
11. J. Peisach, W. E. Blumberg, E. A. Rachmilewitz, *Biochim. Biophys. Acta* **393**, 404 (1975).
12. J. H. Jandl, L. K. Engle, D. W. Allen, *J. Clin. Invest.* **39**, 1818 (1960).
13. D. Drenckhahn, K. Zinke, K. A. Appell, P. S. Low, *Eur. J. Cell Biol.* **34**, 144 (1984).
14. K. C. Appell and P. S. Low, *J. Biol. Chem.* **256**, 11104 (1981).
15. M. M. B. Kay, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5753 (1984).
16. G.Lelkes, G. Lelkes, K. S. Merse, S. R. Hollan, *Biochim. Biophys. Acta* **732**, 48 (1983).
17. H. Müller and H. U. Lutz, *ibid.* **729**, 249 (1983).
18. N. Khansari, G. F. Springer, E. Merler, H. H. Fudenberg, *Mech. Ageing Dev.* **21**, 49 (1983).
19. J. F. Brandts and B. S. Jacobson, *Surv. Synth. Pathol. Res.* **2**, 107 (1983).
20. T. L. Steck, *J. Supramol. Struct.* **8**, 311 (1978).
21. E. Schweizer, W. Angst, H. U. Lutz, *Biochemistry* **21**, 6807 (1982).
22. P. S. Low *et al.*, in preparation.
23. J. R. Murphy, *J. Lab. Clin. Med.* **82**, 334 (1973).
24. Supported by a grant from the National Institutes of Health (GM24417) and by a grant from the Deutsche Forschungsgemeinschaft (91-5-1).
* To whom correspondence should be addressed.

11 June 1984; accepted 30 October 1984

Insulin Mediators from Rat Skeletal Muscle Have Differential Effects on Insulin-Sensitive Pathways of Intact Adipocytes

Abstract. *The effects of partially purified insulin-generated mediators from rat skeletal muscle were compared to those of insulin on intact adipocytes. Insulin and insulin mediator stimulated both pyruvate dehydrogenase and glycogen synthase activity of intact adipocytes. In contrast, insulin stimulated glucose oxidation and 3-O-methylglucose transport, whereas insulin-generated mediators had no effect. Insulin-generated mediators cannot account for all the pleiotropic effects of insulin, especially membrane-controlled processes.*

Insulin, as the major anabolic hormone in mammals, is responsible for a variety of effects on numerous metabolic pathways. These pleiotropic responses occur in seconds, minutes, and hours after the interaction of insulin with target cells. The metabolic pathways affected by the hormone involve reactions that are located on the plasma membrane, in cytoplasmic compartments, and in the nucleus. The molecular mechanisms by which insulin causes its effects have not been elucidated. Recent studies have indicated that the interaction of insulin with its receptor causes the release from the plasma membrane of several related, low molecular weight, acid- and heat-stable molecules that appear to function as mediators or second messengers for many of insulin's intracellular effects (*1, 2*). These mediators can be generated by insulin in subcellular systems (*3–11*), from intact cells (*12–14*), and in vivo (*15, 16*). In vitro, these mediators stimulate pyruvate dehydrogenase (*4–7, 9–13, 17*), glycogen synthase (*2, 15, 18*), adenosine 3′,5′-monophosphate (cyclic AMP) phosphodiesterase of low Michaelis constant (*19*), and acetyl coenzyme A carboxylase (*20*) and inhibit adenylate cyclase (*21*), similar to insulin's effects on these same enzymes in vivo.

Recently it was shown that insulin mediator preparations from rat adipocyte and liver plasma membranes affect a variety of metabolic pathways in isolated intact adipocytes (*22*) and hepatocytes (*23*), respectively. In the former system the mediator preparations stimulated lipogenesis and decreased hormonally elevated lipolysis and cyclic AMP levels. In cultured hepatocytes the mediators stimulated lipolysis and caused down-regulation of the insulin receptor. Our laboratory has found that mediator prepared from skeletal muscle of insulin-treated rats stimulated pyruvate dehydrogenase activity of intact adipocytes and that the mediator was degraded by the adipocyte with time (*24*). The ability of these mediator preparations to act on intact cellular systems opens new avenues for investigating the effects of these mediators on various metabolic pathways not approachable in subcellular systems.

In this study we compared the effect of insulin and of partially purified preparations of insulin mediator from rat skeletal muscle on glucose oxidation, 3-*O*-methylglucose transport, pyruvate dehydrogenase activity, and glycogen synthase activity by isolated rat adipocytes. This approach allows investigation of insulin-sensitive membrane processes, such as glucose transport, that cannot be studied in subcellular systems.

The mediators used were extracted from freeze-clamped skeletal muscle from control and insulin-treated rats with a mixture of 0.2*M* sodium acetate buffer (*p*H 3.8), 1 m*M* EDTA, and 0.1 m*M* dithiothreitol (*16*). The extract was centrifuged in the cold to remove cellular debris, boiled, and ultracentrifuged to remove denatured material. Nucleotide impurity was extracted with charcoal and the supernatant was sized by ultrafiltration. The fraction of molecular weight 500 to 2000 was lyophilized and taken up in 1 m*M* formic acid (4 ml per 10 g of original tissue weight). This procedure provided a 500-fold purification from starting material on the basis of protein measurement. Adipocytes were prepared by a modification of the method of Rodbell (*25*). The cells were incubated and enzyme activity or glucose oxidation was measured as described in the legends to Figs. 1 to 3.

The effects of insulin and mediators from muscle of control or insulin-treated rats on intact rat adipocyte pyruvate dehydrogenase are illustrated in Fig. 1. Insulin at 100 μU/ml stimulated pyruvate dehydrogenase activity twofold, which was the maximal response to the hormone. This stimulation is comparable to that measured by other investigators us-

ing a similar system (*26*). Mediator from muscle of control rats also caused a twofold stimulation, while mediator from muscle of insulin-treated rats caused an almost fourfold stimulation of pyruvate dehydrogenase activity.

The data in Fig. 2 demonstrate the effect of insulin and of insulin mediator on glycogen synthase activity in intact rat adipocytes. Insulin caused a twofold stimulation of glycogen synthase, comparable to that reported by Lawrence and Larner (*27*). The control mediator preparation caused only a slight, but not significant, increase in glycogen synthase activity. In contrast, the mediator from insulin-treated rats caused a twofold stimulation of glycogen synthase activity, similar to the effect of insulin on the enzyme system in adipocytes (*27*) and to insulin mediator from skeletal muscle of rabbits on glycogen synthase activity in vitro (*18*).

Figure 3 compares the effects of control and insulin mediator preparations to that of insulin on glucose oxidation by rat adipocytes. Insulin caused a tenfold stimulation of glucose oxidation. In contrast, neither the mediators from control rats nor insulin-treated rats stimulated glucose oxidation at a concentration that stimulated pyruvate dehydrogenase and glycogen synthase. Since the amount of glucose oxidized by the rat adipocyte indirectly reflects the rate of glucose transport, a rate-limiting step for glucose oxidation, it can be inferred that the insulin mediator does not affect glucose transport. This possibility was directly tested by measuring the effect of the mediator preparations on 3-*O*-methylglucose transport of adipocytes (*28*). Adipocytes were incubated for 10 minutes in the absence or presence of insulin or mediators and transport was measured for 10 seconds. Whereas insulin stimulated transport (177 ± 11 percent of baseline; $n = 14$), neither mediators from control rats (107 ± 3 percent; $n = 7$) nor insulin-treated rats (109 ± 4 percent; $n = 7$) had any significant effect.

In this study we took advantage of the ability of the putative insulin mediators to elicit responses in intact cell systems in an effort to delineate their physiological role in insulin-sensitive membrane and intracellular reactions. We previously showed that partially purified preparations of insulin mediator from skeletal muscle stimulate pyruvate dehydrogenase activity of isolated adipocytes and that adipocytes metabolize the pyruvate dehydrogenase–stimulating mediator (*24*). Not only was it confirmed in this study that the mediators stimulate pyruvate dehydrogenase activity, it was demonstrated that an insulin mediator stimulates glycogen synthase activity in the intact cell system. Interestingly, the mediator from muscle of control rats had little if any effect on glycogen synthase activity, whereas the mediator from insulin-treated rats stimulated the enzyme to the same extent as insulin. In contrast, the control mediator at the same concentration stimulated pyruvate dehydrogenase activity to the same extent as insulin, with the insulin mediator causing twice the stimulation as insulin. These findings support the suggestion that the mediator that stimulates pyruvate dehydrogenase differs from the one that stimulates glycogen synthase and inhibits adenylate cyclase, with both being part of a family of insulin-generated mediators (*1, 2, 18*).

The insulin-generated mediators that work on various intracellular insulin-sensitive enzyme systems cannot account for all the pleiotropic effects of insulin on target cells. The mediators had no effect on glucose oxidation or 3-*O*-methylglucose transport under the same conditions in which the mediators stimulated pyruvate dehydrogenase and glycogen syn-

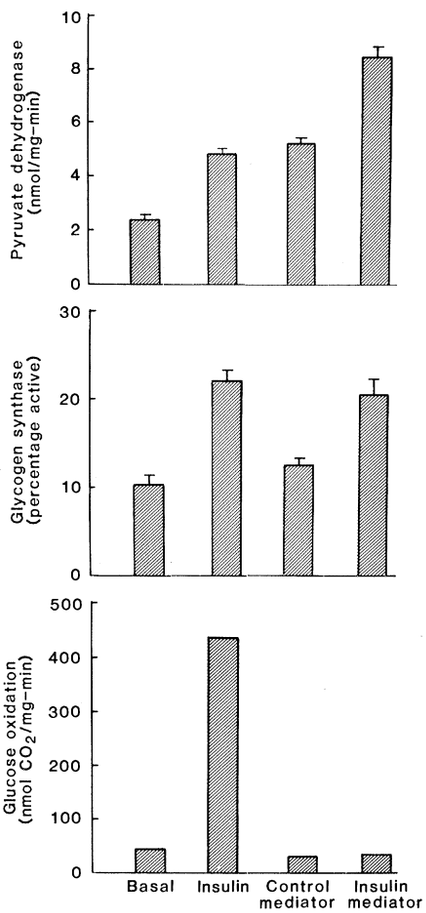

Fig. 1 (top). Effect of insulin and insulin mediator on pyruvate dehydrogenase activity of intact adipocytes. Adipocytes (3.2×10^5 cells) were dispensed into plastic vials and incubated for 15 minutes in Krebs-Ringer phosphate buffer containing 10 m*M* $NaHCO_3$ and 3 percent bovine serum albumin at 37°C with insulin (100 μU/ml) or mediators (1 to 20 dilutions) from control or insulin-treated rats in a total volume of 1.2 ml. One milliliter of the cell suspension was microcentrifuged for 4 seconds and the infranatant was discarded. The cell layer was extracted with 0.5 ml of ice-cold solution containing 50 m*M* KH_2PO_4, 0.2 percent Triton X-100, 2 m*M* dithiothreitol, 2 m*M* EDTA, and 2 m*M* EGTA (*p*H 7.4). The extract was further vortexed, microcentrifuged, and chilled, and the lipid-free infranatant was removed. Pyruvate dehydrogenase activity in the infranatant containing approximately 0.03 mg of total cell protein was assayed as the release of $^{14}CO_2$ from [1-^{14}C]pyruvic acid (*32*). Data are means ± standard errors of triplicate adipocyte incubations performed in the same experiments. Similar results were obtained with other mediator preparations in five other experiments. Fig. 2 (center). Effect of insulin and insulin mediator on glycogen synthase activity of intact adipocytes. Adipocytes (1.0×10^6 cells per milliliter) in Krebs-Ringer bicarbonate buffer containing 3 percent bovine serum albumin (*p*H 7.4) were incubated in plastic vials for 20 minutes at 37°C. Insulin (100 μU/ml) or mediator (1 to 20 dilutions) from control and insulin-treated rats was added and the cells were incubated for an additional 15 minutes. Incubation was stopped by adding 1 ml of fat cell suspension to 0.5 ml of 50 m*M* TES [*N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid], 100 m*M* NaF, 10 m*M* EDTA, and 5 m*M* 2-mercaptoethanol (*p*H 7.4) at 0°C. The cells were microcentrifuged for 4 seconds, the infranatant was discarded, and the cells were rewashed in the same buffer. The cells were resuspended in 0.5 ml of 50 m*M* TES, 25 m*M* NaF, 5 m*M* EDTA, and 5 m*M* 2-mercaptoethanol (*p*H 7.4) and were then sonicated. Cellular debris were removed by centrifugation. Glycogen synthase activity was determined in the supernatant fractions by slight modifications of previously described methods (*33*). Since there was no effect of any addition on total glycogen synthase activity (30.5 ± 1.29 nmol/min per 10^6 cells), results are expressed as active glycogen synthase (determined in the presence of 10 m*M* Na_2SO_4) as a percentage of total glycogen synthase activity (determined in the presence of 6.7 m*M* glucose-6-phosphate). Data shown are means ± standard errors of triplicate adipocyte incubations performed in the same experiment. Similar results were obtained with other mediator preparations in three other experiments. Fig. 3 (bottom). Effect of insulin and insulin mediator on glucose oxidation by intact adipocytes. Adipocytes (8×10^4 to 9×10^4 cells per milliliter) in Krebs-Ringer phosphate buffer with 3 percent bovine serum albumin (*p*H 7.4) were incubated without shaking at 37°C for 15 minutes; 0.75-ml portions were then dispensed into plastic scintillation vials containing insulin (100 μU/ml) or mediator (1 to 20 dilutions) from control or insulin-treated rats [1-^{14}C]D-glucose (0.4 μCi) and D-glucose (0.83 mmol). The cells were incubated for 60 minutes at 37°C with gentle shaking. Glucose oxidation was determined as the amount of $^{14}CO_2$ released (*34*). Data are means of triplicate adipocyte incubations performed in the same experiment (standard errors were too small to illustrate). Similar results were obtained with other mediator preparations in three other experiments.

thase. These findings support the concept that insulin mediators are involved in the rapid intracellular responses to insulin but probably are not involved in membrane transport processes. Consistent with this concept is the finding of Gelehrter *et al.* (*29*) that the mechanism responsible for insulin stimulation of ion transport in rat hepatoma cells differs from the postbinding mechanisms involved in the stimulation of amino acid transport and tyrosine aminotransferase induction.

Two recent reports would indicate that insulin receptor phosphorylation is not involved in the mechanism responsible for insulin stimulation of glucose transport. Plehwe *et al.* (*30*) showed that marked suppression of insulin-stimulated phosphorylation had little if any effect on insulin-stimulated glucose transport. Simpson and Hedo (*31*) reported that an antiserum to the insulin receptor mimicked insulin's action on glucose transport, phosphorylation of integral membrane proteins, and internalization of the insulin receptor in fat cells, while having no effect on phosphorylation of the subunit of the insulin receptor. Thus, yet to be defined mechanisms must be involved in insulin stimulation of glucose transport. Experiments involving subcellular systems, intact cells, and whole animals will be needed to define the exact role of the insulin mediators in each of insulin's actions. At the same time, major efforts are needed to purify and chemically identify this apparently new family of mediator substances.

LEONARD JARETT
Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia 19104

ELLEN H. A. WONG
Department of Biochemistry, National University of Singapore, Kent Ridge, Singapore 0511

S. LANCE MACAULAY
JUDITH A. SMITH
Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine

References and Notes

1. L. Jarett, F. L. Kiechle, J. C. Parker, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 273 (1982); L. Jarett, F. L. Kiechle, J. C. Parker, S. L. Macaulay, *Am. J. Med.* **74**, 31 (1983); J. Larner, *J. Cyclic Nucl. Res.* **8**, 289 (1982); J. R. Seals and M. P. Czech, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 2730 (1982).
2. J. Larner, *Am. J. Med.* **74**, 38 (1983).
3. J. R. Seals, J. M. McDonald, L. Jarett, *J. Biol. Chem.* **254**, 6991 (1979); *ibid.*, p. 6997.
4. L. Jarett and J. R. Seals, *Science* **206**, 1407 (1979).
5. J. R. Seals and L. Jarett, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 77 (1980).
6. J. R. Seals and M. P. Czech, *J. Biol. Chem.* **255**, 6529 (1980).
7. F. L. Kiechle, L. Jarett, D. A. Popp, N. Kotagal, *ibid.* **256**, 2945 (1981).
8. A. Saltiel, S. Jacobs, M. Siegel, P. Cuatrecasas, *Biochem. Biophys. Res. Commun.* **102**, 1041 (1981).
9. Y. Sakamoto, T. Kuzuya, J. Sato, *Biomed. Res.* **3**, 599 (1982).
10. N. Begum, H. M. Tepperman, J. Tepperman, *Endocrinology* **110**, 1914 (1982).
11. J. M. Amatruda and C. L. Chang, *Biochem. Biophys. Res. Commun.* **112**, 35 (1983).
12. F. L. Kiechle, L. Jarett, D. Popp, N. Kotagal, *Diabetes* **29**, 852 (1980).
13. J. C. Parker, F. L. Kiechle, L. Jarett, *Arch. Biochem. Biophys.* **215**, 339 (1982).
14. L. Jarett, F. L. Kiechle, D. A. Popp, N. Kotagal, J. R. Gavin III, *Biochem. Biophys. Res. Commun.* **96**, 735 (1980).
15. J. Larner *et al.*, *Science* **206**, 1408 (1979).
16. J. C. Parker and L. Jarett, *Diabetes*, in press.
17. J. R. Seals and M. P. Czech, *ibid.*, p. 2894; D. Popp, F. L. Kiechle, N. Kotagal, L. Jarett, *J. Biol. Chem.* **255**, 7540 (1980).
18. J. Larner *et al.*, *Recent Prog. Horm. Res.* **38**, 511 (1982).
19. F. L. Kiechle and L. Jarett, *FEBS Lett.* **133**, 279 (1981).
20. A. R. Saltiel, A. Doble, S. Jacobs, P. Cuatrecasas, *Biochem. Biophys. Res. Commun.* **110**, 789 (1983).
21. A. R. Saltiel, M. I. Siegel, S. Jacobs, P. Cuatrecasas, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3513 (1982).
22. S.-R. Zhang, Q.-H. Shi, R. J. Ho, *J. Biol. Chem.* **258**, 6471 (1983).
23. J. F. Caro, F. Folli, F. Cecchin, M. K. Sinha, *Biochim. Biophys. Res. Commun.* **115**, 375 (1983).
24. L. Jarett, E. H. A. Wong, J. A. Smith, S. L. Macauley, *Endocrinology*, in press.
25. M. Rodbell, *J. Biol. Chem.* **239**, 375 (1964).
26. L. Mukherjee and R. L. Jungas, *Biochem. J.* **148**, 229 (1975); R. M. Denton and W. A. Hughes, *Int. J. Biochem.* **9**, 545 (1978); J. M. May and C. de Haan, *J. Biol. Chem.* **254**, 9017 (1979); S. P. Mukherjee and C. Mukherjee, *Arch. Biochem. Biophys.* **214**, 211 (1982).
27. J. C. Lawrence, Jr., and J. Larner, *Mol. Pharmacol.* **14**, 1079 (1978).
28. C. Ludvigsen, L. Jarett, J. M. McDonald, *Endocrinology* **106**, 786 (1980).
29. T. D. Gelehrter *et al.*, *Diabetes* **33**, 428 (1984).
30. W. E. Plehwe *et al.*, *Biochem. J.* **214**, 361 (1983).
31. I. A. Simpson and J. A. Hedo, *Science* **223**, 1301 (1984).
32. F. L. Kiechle and L. Jarett, *Mol. Cell. Biochem.* **56**, 99 (1983).
33. J. A. Thomas, K. K. Schlender, J. Larner, *Anal. Biochem.* **25**, 486 (1968); *Biochim. Biophys. Acta* **193**, 84 (1973).
34. L. Jarett and R. M. Smith, *J. Biol. Chem.* **249**, 7024 (1974).
35. Supported in part by NIH grants AM28144 and AM19525 and a grant from the Reynolds Foundation. S.L.M. is supported by a grant from the Juvenile Diabetes Foundation and E.H.A.W. is a fellow of the American Association of University Women. We acknowledge the technical contribution of J. O. Macaulay.

16 July 1984; accepted 7 December 1984

Vaccination Against *Schistosoma mansoni* with Purified Surface Antigens

Abstract. *Two surface antigens were isolated from young or adult schistosomes by affinity chromatography with monoclonal antibodies. Vaccination with an antigen having a molecular weight of 155,000 gave partial protection against challenge in some batches of mice and in a group of cynomolgus monkeys. Vaccination with an antigen having a molecular weight of 53,000 gave similar levels of protection in mice. The results demonstrate that protection can be obtained with single antigens, but the precise requirements for reproducible vaccination are as yet unknown.*

Schistosomiasis is a debilitating tropical parasitic disease that affects more than 200 million people in 73 countries. The causal agents are small paired worms that live in the hepatic portal system and produce large numbers of eggs, which escape via the intestinal wall and feces and hatch in fresh water. The miracidial stage that emerges from the egg infects certain species of snails and multiplies asexually in this intermediate host. Man is infected by direct penetration of the skin by the cercarial stage, which emerges from the snail into water.

An infection can induce substantial, though not complete, resistance against reinfection in several laboratory hosts, and there is circumstantial evidence that immunity develops in man (*1*, *2*). Vaccination with large numbers of living juvenile schistosomes, irradiated to restrict development, can stimulate high levels of protection (*3*), but most attempts to vaccinate with extracted material have been only marginally effective (*4*). One unconfirmed report indicates that extracts of cercariae, the infective stage, can give significant protection in mice (*5*). In vitro studies and experiments with irradiated cercariae have shown that the target antigens are located on the surface membrane of the young schistosomula stage, which is formed as soon as the cercariae penetrate the skin (*2*). There is considerable interest in characterizing individual surface antigens with monoclonal antibodies (*6*), and several groups are moving toward the production of target antigens by genetic engineering in order to obtain sufficient quantities for vaccination studies (*7*). Although large-scale production of antigens will almost certainly require biosynthesis or synthesis of short peptides, quantities sufficient for experimental vaccination can be obtained from moderate numbers of schistosomulae or adult schistosomes. We report here the isolation of two surface antigens by affinity chromatography with monoclonal antibodies and present preliminary results of vaccination trials.

Eight mouse monoclonal antibodies directed against surface antigens of schistosomulae were available, but only one of these, an immunoglobulin M (IgM) antibody (WP66.4), mediates a sig-