25. M. W. Verghese, C. D. Smith, R. Snyderman, in preparation.
26. S. A. Orellana, P. A. Solski, J. P. Brown, *J. Biol. Chem.* **260**, 5236 (1985).
27. S. E. Rittenhouse and J. P. Sasson, *ibid.*, p. 8657.
28. M. J. Berridge, *Biochem. J.* **212**, 849 (1983).
29. M. Goodhart, N. Ferry, P. Geynet, J. Hanoune, *J. Biol. Chem.* **257**, 11577 (1982).
30. E. C. Hulme, C. P. Berrie, N. J. M. Birdsall, A. J. V. Burgen, in *Drug Receptors and their Effectors*, N. J. M. Birdsall, Ed. (Macmillan, London, 1981), pp. 23–34.
31. P. H. Naccache, M. M. Molski, M. Volpi, E. L. Becker, R. I. Sha'afi, *Biochem. Biophys. Res. Commun.* **130**, 677 (1985).
32. P. J. Stein, M. M. Rasenick, M. W. Bitensky, in *Progress in Retinal Research*, N. Osborne and G. Chadler, Eds. (Pergamon, New York, 1982), pp. 227–243.
33. PMN's (>95 percent purity) were isolated from blood obtained from healthy donors as described (*12*). Plasma membranes were prepared by N_2 cavitation and subsequent centrifugation for 60 minutes at 90,000*g* over a cushion of 41 percent sucrose [T. Maeda, K. Balakrishnan, S. Q. Mehdi, *Biochim. Biophys. Acta* **731**, 115 (1983)]. The membranes banding at the interface were recovered by centrifugation at 150,000*g* for 30 minutes, washed with 0.25*M* sucrose containing 10 m*M* Hepes buffer (*p*H 7.0), and then resuspended in the same buffer. Membranes were used immediately or frozen at −80°C. A single freeze-thaw cycle did not disrupt the coupling of the receptor and PIP_2 hydrolysis.
34. J. Schacht, *Methods Enzymol.* **72**, 626 (1981).
35. The acidified chloroform:methanol solutions were centrifuged, and the aqueous phase was neutralized with NaOH and diluted to 20 ml with water, producing a final salt concentration of approximately 50 m*M*. The entire sample was then chromatographed on a 0.5-ml column of Dowex 1 (formate), eluted with 5 ml of the buffers described (*28*). The samples were then combined with 12 ml of Lefko-Fluor scintillation mixture and analyzed by liquid scintillation spectrometry.
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Anti-Idiotypic Antibodies Bear the Internal Image of a Human Tumor Antigen

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Goat antibodies to idiotypes (anti-idiotypic antibodies; Ab2) that recognize an idiotype associated with the combining site of a BALB/c mouse IgG2a monoclonal antibody (Ab1) to human gastric carcinoma were used to immunize BALB/c mice and rabbits. A monoclonal anti–anti-idiotypic antibody (Ab3) of IgG1 isotype was obtained after immunization of mice. The Ab3 and the Ab1 showed identical binding specificities and bound with similar avidities to the same tumor antigen. The induction of Ab1-like Ab3 by Ab2 was not restricted to mice, since Ab3 could also be induced in rabbits. Both the mouse- and the rabbit-derived Ab3 bound the same gastrointestinal cancer-associated antigen as Ab1. These findings indicate that Ab2 induced the formation of antigen-specific Ab3, probably because it bears the internal image of the tumor-associated antigen. This Ab2 may therefore have potential for modulating the immune response of cancer patients to their tumors.

WE HAVE PREVIOUSLY DISCUSSED (*1, 2*) the possibility of a beneficial role of antibodies to idiotypes (anti-Id) induced in patients with gastrointestinal tract tumors after treatment with a monoclonal antibody (MAb). Direct evidence for a beneficial effect of anti-Id would be the triggering of an anti-tumor immune response in cancer patients after administration of anti-Id. Of the various populations of anti-Id directed against anti-tumor antibodies, those bearing the internal image of the tumor antigen will selectively stimulate antigen-specific B and/or T cells and will induce immunity in various species. Induction of antigen-specific immunity by anti-Id that most likely bear the internal image of the antigen has been reported in the rabbit allotype (*3*) and tobacco mosaic virus (*4*) systems. Immunization with anti-Id may play an important role in those cases where the antigen is not available. Since cancer antigens are not available in quantities sufficient for immunization, we have investigated the possibility that an internal image of the cancer antigen carried by an immunoglobulin (anti-idiotype) may produce an immune response against the tumor.

MAb GA733 (referred to hereafter as Ab1), with binding specificity for human carcinomas of various tissue origins, was chosen for production of anti-Id because of its tumoricidal activity in vitro and in vivo in nude mice (*5*). Tumor-associated antigen was purified from tumor cell lysates on an Ab1-linked affinity column (*6*). Anti-Id (Ab2) was produced in goats and isolated from the sera as described (*7*). The anti-idiotypic nature of affinity-purified goat Ab2 has been described previously (*2, 7*). The Ab2, at a concentration of 0.2 μg/ml, inhibited binding of iodinated Ab1 to target cells by 97 percent, indicating that Ab2 is directed to one or more epitopes within or near the combining site of Ab1. In control experiments, Ab2 did not significantly inhibit binding of an unrelated immunoglobulin G (IgG2a) MAb to the same target cells. The Ab2 population with combining site–related specificity represented approximately 67 percent of the total Ab2 population as determined by inhibition of binding of Ab2 to Ab1 by GA733 tumor-associated antigen. In a control, we observed no significant inhibition of binding of Ab2 to Ab1 GA733 in the presence of human serum albumin.

These results suggested that a major pop-

Table 1. Characterization of Ab1 (MAb GA733) and Ab3 CE5. Ab1 was generated as described (*5*). To generate Ab3, BALB/c mice were immunized with 50 μg of Ab2 in complete Freund's adjuvant subcutaneously and were injected on day 11 with the same amount of Ab2 in incomplete Freund's adjuvant, and again intravenously without adjuvant on day 33. Three days later, spleen cells were fused with non-secretor 653 mouse myeloma cells (*8*). Antibody isotype was determined by Ouchterlony immunodiffusion. Binding of ^{125}I-labeled GA733 antigen to either Ab1 or Ab3 was tested in a solid-phase radioimmunoassay (*18*). Antibody avidity and number of antibody-binding sites per cell were determined by Scatchard analysis (*19*). ADMC against [^{3}H]thymidine-labeled CRC cells was determined as described, by means of thioglycollate-elicited murine peritoneal macrophages as effector cells (*9*). Inhibition of binding of ^{125}I-labeled Ab1 to Ab2 by Ab3 or Ab1 was measured by a solid-phase radioimmunoassay (*18*) in which Ab2 at a concentration of 0.5 μg/ml was bound to wells of polyvinyl microtiter plates and then the wells were incubated with various concentrations of Ab3 CE5 or Ab1 GA733. Finally, the wells were incubated with ^{125}I-labeled Ab1 (20,000 count/min per well) and inhibition of binding of ^{125}I-labeled Ab1 was calculated.

Parameter investigated	Ab1 (MAb GA733)	Ab3 CE5
Isotype	IgG2a	IgG1
Binding to ^{125}I-labeled GA733 antigen (count/min)	10,905.0	1,410.0
Binding avidity (association constant $K_a \times 10^7\ M^{-1}$) ± SEM	2.0 ± 1.0	4.0 ± 1.0
Number of Ab-binding sites per cell ($\times 10^6$) ± SEM	3.0 ± 0.5	2.7 ± 0.1
ADMC (% specific lysis)	57.4	25.1
Maximum inhibition of binding of Ab1 to Ab2 (%)	95.6	41.0

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ulation of the Ab2 might bear the internal image of the GA733 antigen. To confirm these findings, we immunized BALB/c mice (the strain used for generation of MAb GA733) with Ab2 (Table 1) for production of anti–anti-idiotypic antibodies (Ab3) that might share idiotopes with Ab1 and exhibit identical binding specificity. Monoclonal Ab3 were generated after fusion of spleen cells from immunized mice with non-secretor 653 mouse myeloma cells (*8*). From one BALB/c mouse, 120 hybridoma colonies were derived. Eight of the 120 colonies produced antibodies that bound in preliminary radioimmunoassay screening to SW948 colorectal carcinoma cells (CRC) expressing GA733 antigen. Cells from one of the eight wells were cloned twice, and MAb secreted by a subclone (CE5) were purified on protein A–Sepharose columns (Bio-Rad) according to the manufacturer's directions. Both purified Ab3 CE5 and Ab1 bound the isolated GA733 antigen (Table 1). Since the binding avidities to intact cells and the number of antigenic sites per cell were similar for both antibodies, the less efficient binding of Ab3 CE5 to isolated GA733 antigen may indicate that the interaction of this antibody with antigen may be more sensitive to detergent solubilization of the antigen. Ab3 CE5 was less effective than Ab1 in antibody-dependent macrophage-mediated cytotoxicity (ADMC) assays (*9*). This finding is in agreement with previous results (*9, 10*) which showed that MAb of IgG1 isotype was much less effective in ADMC than IgG2a MAb.

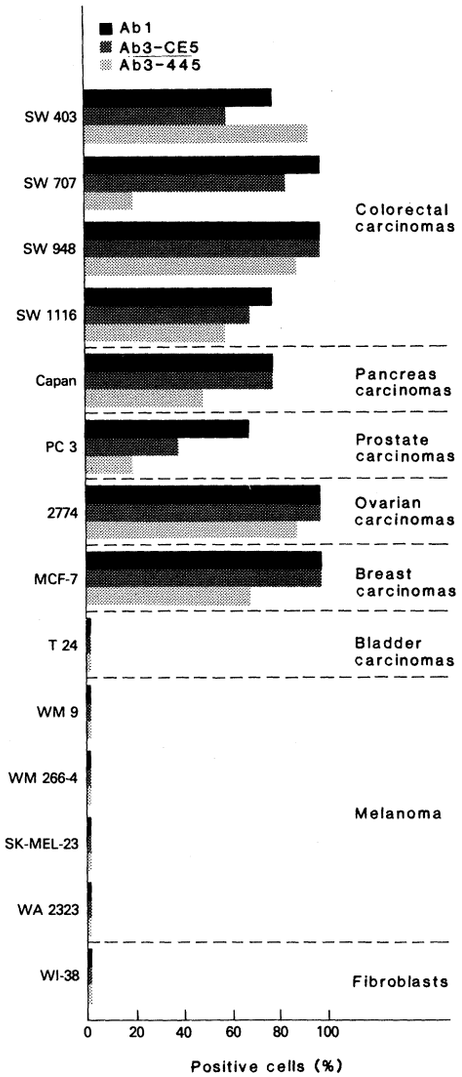

Fig. 1. Immunoreactivity of murine monoclonal Ab1 GA733, Ab3 CE5, and rabbit polyclonal Ab3 445. Generation of murine MAb is as described in the legend to Table 1. A New Zealand white rabbit (445) was injected subcutaneously with 300 μg of Ab2 in complete Freund's adjuvant on day 0 and 100 μg of Ab2 in incomplete adjuvant on day 7, followed by two intramuscular injections each of 100 μg of Ab2 in saline on days 21 and 64. The rabbit was bled 14 days after the last injection. Percentage of antibody-reactive cells was determined by MHA as described for murine MAb (*11*). Binding of rabbit antibodies to tumor cells was detected in a modified MHA by means of an indicator system of sheep red blood cells (SRBC) coated with rabbit anti-SRBC antibody to which goat anti-rabbit IgG had been bound.

To investigate whether induction of Ab1-like Ab3 was species-restricted, rabbits were also immunized with Ab2 to generate polyclonal Ab3. Sera from the two rabbits inhibited binding of iodinated Ab1 to Ab2 by 67 and 69 percent, respectively, indicating the presence of Ab3 sharing idiotopes with Ab1. No significant inhibition by preimmune sera was observed. These inhibition assays were performed in the presence of 5 percent normal goat serum to minimize steric hindrance of the combining region of Ab2 by anti-isotypic and anti-allotypic antibodies present in the rabbit sera. Sera from both rabbits bound to various target cells in a pattern identical to that of Ab1 as demonstrated for serum of rabbit 445 (Ab3 445) in Fig. 1.

Since the GA733 antigen has been identified and characterized (*5*), it was of interest to determine whether the mouse-derived Ab3 CE5 and the rabbit-derived Ab3 would precipitate the same glycoprotein. The results (Fig. 2A) indicate that both Ab1 and Ab3 CE5 precipitated the iodinated GA733 antigen. The same antigen was precipitated by both antibodies from cell extracts that had been prepared from metabolically labeled CRC cells (*12*). To test for the interaction of rabbit-derived Ab3 with GA733 antigen, antigen-specific Ab3 was isolated from the serum of rabbit 447 by adsorption of the serum to glutaraldehyde-fixed SW948 CRC cells that expressed GA733 antigen;

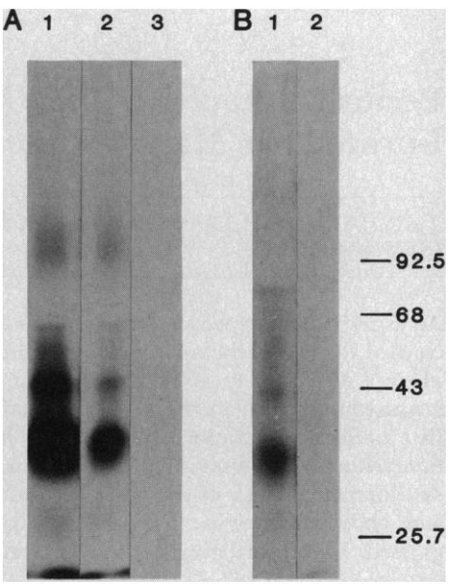

Fig. 2. (A) Electrophoretic profile of ^{125}I-labeled GA733 antigen after immunoprecipitation with purified Ab1 (lane 1), Ab3 CE5 (lane 2), or unrelated MAb ME7529 (lane 3). Marker sizes are shown in kilodaltons (kD). The major band at 30 kD and the minor band at 40 kD represent the ^{125}I-labeled GA733 antigen. All antibodies were used at a concentration of 2.7 μg/ml. GA733 antigen was isolated on Ab1 affinity columns (*6*) and labeled with ^{125}I by the chloramine-T method (*20*); it was incubated with MAb and then with goat anti-mouse IgG antibody coupled to agarose beads. The precipitated molecules were analyzed by gel electrophoresis and autoradiography. The film was exposed for 3 days. (B) Electrophoretic profile of ^{125}I-labeled GA733 antigen bound to Ab3 447 that had been isolated from serum by adsorption to SW948 CRC cells and elution of bound antibody with glycine HCl buffer, *p*H 2.8 (lane 1); absence of binding to normal rabbit IgG (lane 2). Ab3 447 and normal rabbit IgG were used at concentrations of 8 and 25 μg/ml, respectively. The film was exposed for 10 days.

the bound antibody was then eluted with 0.1*M* glycine HCl buffer (*p*H 2.8). Isolated rabbit Ab3 447 bound the same antigen that was precipitated by Ab1 (Fig. 2, A and B).

Kennedy *et al.* (*13*) have reported inhibition of a virus-induced tumor by anti-Id directed against an idiotope of a single MAb to SV40 T antigen; this idiotope was not expressed by other MAb's reacting with T antigen. In addition, the modulation of the immune response to tumors after administration of anti-Id has been observed (*14–16*). Our conclusion that Ab2 may carry the internal image of the GA733 tumor-associated antigen is based on the following observations: (i) Ab2 induced the formation of antigen-specific Ab3 in the absence of exposure to antigen in two different species of animals; (ii) Ab2 inhibited binding of Ab1 to target cells; and (iii) the binding of Ab1 to Ab2 was inhibited by tumor antigen. We have also shown that Ab3 CE5 mediated cytolytic effects on human tumor cells in culture. Once IgG2a-producing isotypic switch-variant hybridoma cells are isolated from the IgG1-producing Ab3 CE5 hybridoma cells (*17*), it will be possible to demonstrate tumoricidal activity of Ab3 CE5 in nude mice containing human tumor xenografts (*9*). The beneficial role of anti-Id in cancer patients can then be directly investigated by clinical studies of patients that have been immunized with anti-Id to induce humoral and/or cellular immune responses.

REFERENCES AND NOTES

1. H. Koprowski, D. Herlyn, M. Lubeck, E. DeFreitas, H. F. Sears, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 216 (1984).
2. E. DeFreitas *et al.*, *Curr. Top. Microbiol. Immunol.* **119**, 75 (1985).
3. P. A. Cazenave and J. Roland, *Immunol. Rev.* **79**, 139 (1984).
4. J. Urbain, M. Slaoui, B. Mariame, O. Leo, in *Idiotypy in Biology and Medicine*, H. Köhler, J. Urbain, P. A. Cazenave, Eds. (Academic Press, New York, 1984) p. 15.
5. D. Herlyn *et al.*, *J. Immunol. Methods* **73**, 157 (1984).
6. A. H. Ross, D. Herlyn, D. Iliopoulos, H. Koprowski, *Biochem. Biophys. Res. Commun.*, in press.
7. D. Herlyn, M. Lubeck, H. F. Sears, H. Koprowski, *J. Immunol. Methods* **85**, 27 (1985).
8. J. F. Kearney, A. Radbruch, B. Liesegang, *J. Immunol.* **123**, 1548 (1979).
9. D. Herlyn and H. Koprowski, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4761 (1982).
10. D. Herlyn, M. Herlyn, Z. Steplewski, H. Koprowski, *Cell. Immunol.* **92**, 105 (1985).
11. M. Herlyn *et al.*, *Cancer Res.* **40**, 3602 (1980).
12. A. H. Ross, G. Cossu, J. R. Bell, Z. Steplewski, H. Koprowski, *Arch. Biochem. Biophys.* **225**, 370 (1983).
13. R. C. Kennedy, G. R. Dreesman, J. S. Butel, R. E. Lanford, *J. Exp. Med.* **161**, 1432 (1985).
14. J. W. Forstrom, K. A. Nelson, G. T. Nepom, I. Hellstrom, K. E. Hellstrom, *Nature (London)* **303**, 627 (1983).
15. G. T. Nepom, K. A. Nelson, S. L. Holbeck, I. Hellstrom, K. E. Hellstrom, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2864 (1984).
16. V. K. Lee *et al.*, *ibid.*, **82**, 6286 (1985).
17. Z. Steplewski *et al.*, *ibid.*, p. 8653.
18. H. Koprowski, M. Herlyn, Z. Steplewski, H. F. Sears, *Science* **212**, 53 (1981).
19. D. Herlyn, J. Powe, A. H. Ross, M. Herlyn, H. Koprowski, *J. Immunol.* **134**, 1300 (1985).
20. A. H. Ross *et al.*, *Arch. Biochem. Biophys.* **242**, 540 (1985).

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Perturbation of Red Cell Membrane Structure During Intracellular Maturation of *Plasmodium falciparum*

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An experimental approach, which in this study was applied to the malarial system, can be used to analyze the molecular structure and organization of individual phospholipids in a wide variety of biological membranes. Electron spin resonance spectroscopy was used to investigate the structural modifications of the major red cell phospholipids that occur in erythrocyte membranes infected with the human malarial parasite, *Plasmodium falciparum*. These modifications were correlated with the intracellular developmental stage of the parasite. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine were increasingly disordered (fluidized) as infection progressed. This disordering occurred at different rates and to varying extents.

THE PATHOPHYSIOLOGIC MANIFESTATIONS of malaria requires invasion of a host red cell and an obligatory intraerythrocytic developmental process. Invasion of the red cell by *Plasmodium falciparum* has been characterized at the ultrastructural level (*1*). The molecular components of the receptor-mediated attachment step are currently being investigated, with major advances reported by Perkins (*2*) and Ginsburg (*3*). However, little is known about the energetics or molecular mechanisms of red cell penetration by the parasite or about the events that occur in the red cell membrane after parasitic invasion. We propose that key events in the invasion and postinvasion processes involve nonuniform alterations in the structure and organization of the major red cell phospholipids. Alterations in the content and organization of the protein and lipid components of the erythrocyte appear as early as the internalization process. Membrane areas rich in rhomboidally arranged intramembranous particles are evident at the parasite-erythrocyte junction, and a presumptive protein-free, lipid-rich environment in the invaginated portion of the red cell membrane has been observed (*4*). The asymmetric arrangement of phospholipids in the uninfected erythrocyte membrane, with phosphatidylcholine (PC) and sphingomyelin localized in the outer monolayer and phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the inner monolayer (*5*), is altered after penetration of the red cell by the parasite (*6*). PE and PS are increased and PC is decreased in the outer monolayer of the infected red cell after invasion. Ginsburg (*3*) characterized new permeation pathways in the infected cells, which appear at the trophozoite stage and are selective for anions. Other membrane alterations include a more negative transmembrane potential in the later stages of intraerythrocytic development (*7*) and the synthesis and insertion of new and altered membrane proteins on the surface of the infected cell (*8*).

It has been suggested (*3*) that modifications of membrane lipid-lipid and lipid-protein interactions play an important role in erythrocyte penetration, intracellular maturation, and release of malarial parasites. There have been, however, no detailed physicochemical analyses of the red cell membrane during these processes. In order to probe membrane structure at the molecular level, we incorporated specific phospholipid spin labels into the native red cell membrane and compared the electron spin resonance (ESR) spectra arising from uninfected erythrocytes to the spectra arising from erythrocytes parasitized with *P. falciparum* at specific stages of development. We report the conformational modifications induced in individual phospholipid classes in the membrane of the infected erythrocyte at different developmental stages of the parasite.

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