hybrid nature of the H1-C4 cell line was confirmed by karyotypic analysis. The H1-C4 cells contained between 129 and 133 chromosomes. Of these chromosomes, 10 to 12 were identified as human metacentric chromosomes, one was identified as the metacentric marker chromosome of the NSI/1 cell, and the rest were acrocentric chromosomes typical of mouse cells.

The production in culture of high-titered human antibodies by transspecies hybrid cells raises the question of whether this technique will have broad application. Although the potential for this method has been recognized (*6, 7*), efforts at developing transspecies hybrids have been hindered by observations that these cells have a high rate of chromosome loss and phenotypic instability. We find, however, that if hybrid cells can be generated at a sufficient frequency, then aggressive selection techniques can compensate for the high loss rate and permit the isolation of the minority population of stable hybrid cells.

Although the majority of our transspecies hybrids were initially phenotypically unstable (transspecies fusions produced 1 to 10 percent as many stable hybrids as intraspecies mouse fusions did), we have now obtained six different stable hybrid cell lines that each produce human monoclonal IgM antibodies. In all of these instances, more than 80 percent of the daughter clones from individual hybrid cell lines showed continued IgM production in culture. Furthermore, several of these stable antibody-producing hybrids have been obtained from fusions that were performed between mouse myeloma cells and nonstimulated human lymphocytes from blood and lymph nodes. It appears therefore that in vitro stimulation with antigen, although useful for the expansion of reactive B cell clones, may not be essential for the generation of successful hybrids.

With increased developments in methods for the selection of antibody-producing hybrids [for example, by use of the fluorescence-activated cell sorter (*17*)] it should be possible to use transspecies hybridization as a general method for the production of human monoclonal antibodies for therapeutic and diagnostic purposes. In this context, studies by Hakomori *et al.* (*18*) have demonstrated the "tumor-specific" expression of Forssman glycolipid in gastric tumors of patients who were apparently Forssman-negative. The selective expression of this glycolipid on tumor cells provides a potential target for immunological intervention. The availability of the H1-C4 human monoclonal antibody against Forssman antigen now permits a more detailed examination of this possibility.

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13. The reactivity of H1-C4 antibody with Forssman antigen was further confirmed by hemagglutination and hemolysis assays. H1-C4 antibody both agglutinated and lysed sheep red blood cells; in parallel assays the antibody did not react with guinea pig or human red blood cells.
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Antibody Targeting of Liposomes: Cell Specificity Obtained by Conjugation of F(ab')$_2$ to Vesicle Surface

Abstract. *A method devised for conjugating liposomes with protein resulted in the binding of up to 200 micrograms of immunoglobulin G per micromole of lipid. The coupling of antibody to human erythrocyte F(ab')$_2$ in vesicles (140 molecules per vesicle) by this method caused a 200-fold increase in the binding of vesicles to human erythrocytes and resulted in about 80 percent of the vesicle lipid and contents being associated with cells.*

Liposomes have gained wide acceptance as potential carriers for introducing drugs and macromolecules into cells (*1*). Several investigators have attempted to increase the extent of liposome interaction with specific cells by inducing specific ligand receptor interactions. Such attempts have included noncovalent association of cell-specific antibodies with liposomes (*2, 3*), coating of liposomes with heat-aggregated immunoglobulin M (IgM) (*4*), lectin-mediated attachment of glycoprotein-bearing liposomes with erythrocytes (*5*), and use of hapten-bearing liposomes and antibody to haptens (*6, 7*). Only two- to sixfold increases in binding were obtained by these methods and a small fraction of the liposomes (up to 5 percent) were affected. Nonspecific association of antibody to vesicles typically involves only 1 to 6 μg of protein per milligram of lipid, and previous attempts to enhance binding by covalent attachment with glutaraldehyde or carbodiimide (*8*) achieved little success. We reported recently a procedure for the attachment of periodate oxidized horseradish peroxidase to liposomes bearing primary amino groups by imine formation and borohydride reduction (*9*). We have now devised an efficient coupling method applicable to many proteins, in which aldehydes on the vesicle surface react with amino groups on the protein (*10*). The aldehyde groups are produced by periodate oxidation of vesicles containing lipids bearing vicinal hydroxyls (glycosphingolipids). With this method up to 200 μg of immunoglobulin G (IgG) is bound per micro-

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mole of lipid and no prior protein modification is required. In this report we describe experiments on the interaction of antibody-linked liposomes with human erythrocytes in which about 80 percent of the targeted vesicles were associated with the cells.

For these experiments, reverse-phase evaporation vesicles (*11*) were prepared from galactocerebroside, phosphatidylglycerol, and cholesterol (in a 45:5:50 mole ratio) or from gangliosides, phosphatidylcholine, and cholesterol (in a 10:45:45 mole ratio) in 10 m*M* borate, and 60 m*M* NaCl buffer, *p*H 8.4. Before the vesicles were coupled to protein they were oxidized by periodate (8 m*M*, *p*H 8.5) for 2 hours at 25°C to produce aldehydes on the liposome surface. Under these conditions, approximately 40 percent of the oxidizable lipid is oxidized (*10*) whereas encapsulated markers, such as sucrose or glycerol-1-phosphate, are neither oxidized nor induced to leak out of the vesicles during the periodate treatment.

These results indicate that periodate oxidizes only the external surface groups without entering the vesicles interior. Excess periodate is readily removed by desalting the vesicles on Sephadex G-75. Protein may be coupled to the oxidized vesicles at *p*H 8.4 with 10 m*M* recrystallized sodium cyanoborohydride (*12*). For these experiments we added 10 mg of $F(ab')_2$ to 10 μmole of vesicle lipid (after oxidation and desalting) in 1 ml of borate-saline (*p*H 8.4), and then added 10 μl of 1*M* sodium cyanoborohydride. After 18 hours at room temperature, the vesicles were separated from unbound protein by flotation on discontinuous dextran gradients (0 to 20 percent, weight to volume). For quantitation of binding to cells the vesicles contained ^{3}H-labeled dipalmitoylphosphatidylcholine (DPPC; 10 μCi/μmole) and ^{14}C-labeled sucrose (1 μCi/μmole). "Targeted" vesicles were coupled to rabbit $F(ab')_2$ to human erythrocytes, and "control" vesicles were coupled to $F(ab')_2$ prepared from rabbit gamma globulin by pepsin digestion and absorption to a *Staphylococcus aureus* suspension.

Human erythrocytes were used for studies of the antibody-mediated binding of the vesicles to the cells. Lipid (1 to 500 nmole) in the form of vesicles was incubated with 10^6 to 10^8 erythrocytes in 0.2 ml of phosphate-buffered saline (PBS) at *p*H 7.4 for 1 hour at 37°C. Cells were washed to remove unbound vesicles (*13*) and were either taken up directly in 10 ml of Triton-toluene scintillant and counted for [^{3}H]DPPC (10^6 to 10^7 cells) or extracted after washing (10^8 cells); the chloroform phase was evaporated and counted for [^{3}H]DPPC content, and the aqueous phase was incubated overnight at 60°C to remove methanol and counted for [^{14}C]sucrose content.

When vesicles were incubated with 10^8 cells there was a marked difference between targeted and control binding (Fig. 1A) with 80 percent of the vesicles binding when 25 to 500 μmole of lipid was added. Control binding with vesicles conjugated to nonspecific $F(ab')_2$ was very low (< 1 percent) and did not appreciably increase between 100 and 500 nmole of added lipid. Both the vesicle lipids and the encapsulated sucrose bound to the cells in nearly identical proportions, indicating that cell binding caused no loss of vesicle contents and that the antibody-conjugated liposome preparation was reasonably homogeneous with respect to lipid, encapsulated aqueous marker, and antibody. When vesicles were incubated with 10^7 erythrocytes there was a marked difference in binding between the targeted and nontargeted samples (Fig. 1B). Although a smaller fraction of the total available

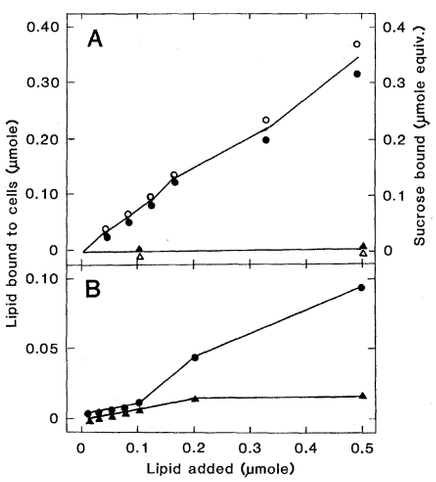

Fig. 1. The binding of targeted vesicles to human erythrocytes. (A) Vesicles were prepared from phosphatidylcholine, cholesterol, and ganglioside (45, 45, and 10 moles, respectively, per 100 moles) and contained 40 μg of rabbit $F(ab')_2$ to human erythrocytes coupled per micromole of lipid (sample) or 66 μg of rabbit $F(ab')_2$ per micromole of lipid (control). Vesicles contained 1 m*M* [^{14}C]sucrose. Symbols: ▲, control lipid ([^{3}H]DPPC); △, control [^{14}C]sucrose; ●, sample lipid; ○, sample sucrose. Lipid binding is expressed as nanomoles of lipid, and sucrose binding as nanomoles of lipid with which it is putatively associated. (B) Vesicles were prepared from galactocerebroside, cholesterol, and phosphatidylglycerol (45, 50, and 5 moles, respectively, per 100 moles) and contained 40 μg $F(ab')_2$ coupled per micromole (sample) or 32 μg of $F(ab')_2$ per micromole (control). Symbols: ●, sample ([^{3}H]DPPC); ▲, control.

vesicles became bound, the number of vesicles that bound to each cell was increased. Vesicles incubated with 10^6 cells also exhibited binding specificity (not shown), with 6 nmole of specific antibody-bearing vesicles and 2 nmole of nonspecific vesicles being bound when 100 nmole of lipid was incubated with the cells. The addition of serum (25 percent fetal calf) during incubation had no substantial effect on the binding.

We measured the hemagglutinating titer of the $F(ab')_2$ conjugated to vesicles and compared it to the original antibody preparation. The nonspecific soluble $F(ab')_2$ and the control vesicles derived from it produced no hemagglutination at concentrations up to 1 mg of $F(ab')_2$ per milliliter. The soluble antibody to human erythrocyte $F(ab')_2$ had a hemagglutinating titer of 4 μg/ml, which is slightly greater than the titer of the conjugated vesicles derived from it (1.5 μg/ml).

The amount of antibody bound is critical for the binding of liposomes to cells. Our vesicle preparations contained 143 molecules per vesicle, assuming $F(ab')_2$ has a molecular weight of 90,000 and that the vesicle preparations contained 1.8×10^{12} vesicles per micromole (for unilamellar vesicles of 0.2 μm diameter). Antibodies not purified immunologically, such as those used here, may contain only 1 to 5 percent of molecules that are specifically reactive to the cell antigens. Our preparation therefore probably contained approximately one to five specific molecules per vesicle, so that most vesicles were specific for the target cells. The use of nonimmunopurified antibodies with coupling methods that bind only a few antibody molecules per vesicle would result in many vesicles having no specificity for the target.

The association of 400 nmole of lipid with 10^8 human erythrocytes constitutes a lipid mass three times greater than the lipid content of the cell membranes. If one assumes that the vesicles are 0.2 μm in diameter and are unilamellar, the number of vesicles bound per cell is 8000 and their encapsulated volume is 0.33 of the cell volume. It is interesting to compare our results with the most rigorous study done to date by Leserman *et al.* (*7*) on the binding of SUV (DNP-Cap-PE) liposomes to MOPC315 cells. In that study, about 0.1 percent of the vesicles bound specifically to the cells, whereas we have achieved 80 percent association. The lipid and vesicle contents bound per cell is 1000 times greater in our experiments (1.3×10^{-12} compared to 4×10^{-9} μmole per cell). Moreover, the human erythrocyte is much smaller than a mouse myeloma cell, and there would

probably be an even greater difference with cells of comparable size. Leserman *et al.* (*7*) expressed reservations concerning the possible efficacy of antibody targeting for intracellular delivery of vesicle contents. While such arguments should be carefully considered, the data from our system suggest that targeted lipid vesicles may be effective in promoting the intracellular delivery of their contents under appropriate conditions. The purity of the antibody, the number of antibody molecules per vesicle, the ratio of internal volume per lipid, the number of vesicles per cell, and the retention of vesicle contents all have important roles in the efficacy of delivery.

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13. Incubations containing 10^6 or 10^7 cells were washed three times by suspending them in 15 ml of PBS and centrifuging the suspension at 1000*g* for 5 minutes. Incubations containing 10^8 cells were washed twice by layering the incubation mixture over 2 ml of 10 percent (weight to volume) dextran (average molecular weight 40,000) and spinning at 3000*g* for 5 minutes to pellet the erythrocytes. The cell pellet was extracted according to the method of E. G. Bligh and W. J. Dyer [*Can. J. Biochem. Physiol.* **37**, 911 (1959)]. Hemagglutination was performed V-shaped microtiter plates (Cooke) with a total volume of 50 μl containing 1 percent (by volume) of human erythrocytes.
14. This work was supported by research grant CA 25526-01 from the National Cancer Institute. R.T.F. is a fellow of the Jane Coffin Child's Memorial Fund for Medical Research. This investigation has been aided by a grant from the Jane Coffin Child's Memorial Fund for Medical Research.

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Adrenocorticotropic Hormone May Be Transported Directly from the Pituitary to the Brain

Abstract. *Experiments were designed to test the hypothesis that pituitary hormones may be delivered directly to the brain. Concentrations of adrenocorticotropic hormone (ACTH) in the plasma were determined in blood samples obtained simultaneously from the carotid artery, the sagittal sinus, and the jugular vein of three awake sheep. Seizures were induced electrically to stimulate ACTH secretion, and at precise intervals thereafter several simultaneous comparisons were made in each animal. In many of the post-seizure comparisons, the ACTH plasma concentrations within the sagittal sinus exceeded those within the carotid artery as well as those within the jugular vein, indicating that this hormone was released from the pituitary and carried directly through capillary beds of brain to the venous blood within the sagittal sinus. The experiment was repeated in one hypophysectomized sheep and, in this animal, ACTH concentration in the plasma was reduced, but that in the sagittal sinus still was elevated after the seizure, an indication that some ACTH (or ACTH-like material) was released from the brain itself.*

Pituitary secretions have long been thought to enter the systemic circulation directly via the cavernous sinus (*1*), but several studies have suggested that some pituitary hormones may be carried directly to the brain (*2–4*). We now report the results of experiments designed to test this hypothesis.

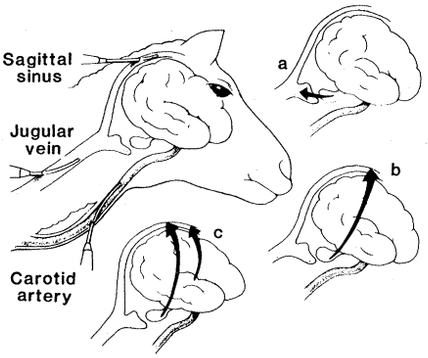

Fig. 1. The experimental model is illustrated. If a pituitary hormone is released into the cavernous sinus, jugular vein concentrations should exceed carotid artery concentrations (a). If a pituitary hormone is carried to the brain and on into the sagittal sinus, the sagittal sinus concentration should exceed the carotid artery concentration (b). Like many other hormones, ACTH is produced by both the brain and the pituitary, and the ACTH found in sagittal sinus venous blood may have come from either source (c). Since ACTH concentrations were diminished by hypophysectomy, most of the ACTH found in high concentration within the sagittal sinus must have come from the pituitary. Yet the persistence of ACTH in sagittal sinus samples after hypophysectomy supports other evidence (*9*) that this hormone is produced and released by the brain as well as the pituitary.

Blood samples were obtained at precisely timed intervals simultaneously from the carotid artery, the sagittal sinus, and the jugular vein of individual sheep; awake and unrestrained animals were used. For each sample, the concentration of adrenocorticotropic hormone (ACTH) was determined by radioimmunoassay. Electrically induced convulsions were used to stimulate ACTH secretion, and during many comparisons the ACTH plasma concentration in the sagittal sinus exceeded that within the carotid artery.

For these experiments three Suffolk sheep were anesthetized and in each animal we placed a single common carotid artery into a subcutaneous loop 10 cm long. After the carotid artery loops were well healed, the animals were anesthetized again; at craniotomy a Silastic catheter (0.16 cm in diameter) was positioned within the sagittal sinus. The catheter was passed forward into the sagittal sinus a distance of 1.0 cm from an insertion point just anterior to the entrance of the vein of Galen (*5*). The catheter was secured to the dura, the scalp, and the wool over the back of the animal, and the craniotomy wound was closed. The next day an indwelling catheter was placed, while the sheep was given a local anesthetic, into the previously prepared ca-

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