A Catalytic Antibody Produces Fluorescent Tracers of Gap Junction Communication in Living Cells*

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The antibody 38C2 efficiently catalyzed a retro-Michael reaction to convert a novel, cell-permeable fluorogenic substrate into fluorescein within living cells. *In vitro*, the antibody converted the substrate to fluorescein with a k_{cat} of 1.7×10^{-5} s^{-1} and a catalytic proficiency ($k_{cat}/k_{uncat}K_m$) of 1.4×10^{10} M^{-1} ($K_m = 7$ μM). For hybridoma cells expressing antibody or Chinese Hamster Ovarian (CHO) cells injected with antibody, incubation of the substrate in the extracellular medium resulted in bright intracellular fluorescence distinguishable from autofluorescence or noncatalyzed conversion of substrate. CHO cells loaded with antibody were 12 times brighter than control cells, and more than 85% of injected cells became fluorescent. The fluorescein produced by the antibody traveled into neighboring cells through gap junctions, as demonstrated by blocking dye transfer using the gap junction inhibitor oleamide. The presence of functional gap junctions in CHO cells was confirmed through oleamide inhibition of lucifer yellow transfer. These studies demonstrate the utility of the intracellular antibody reaction, which could generate tracer dyes in specific cells within complex multicellular environments simply by bathing the system in substrate.

Catalytic antibodies have proven utility for chemical catalysis *in vitro* (1–7). In living cells, these protein catalysts could accomplish a variety of reactions to modify cell behavior or generate reporter molecules for biological assays. Because these antibodies are protein catalysts, the timing and level of expression of such antibodies could be placed under genetic control, making the antibodies potentially valuable markers for studies of cell fate in any situation where single cells must be manipulated in complex multicellular environments. Here we explore whether catalytic antibody reactions can occur within living cells and test their ability to generate sufficient reaction products to be useful in tracing the behavior of individual cells.

A retro-Aldol antibody previously shown to efficiently catalyze numerous reactions was an excellent candidate for the generation of fluorescence *in vivo* (8). Previous work had indicated that fluorescein could be derivatized with side chains, which would both confer membrane permeability and abolish fluorescence (8). We describe a novel fluorogenic substrate bearing side chains that can be cleaved by the antibody-catalyzed reaction to generate fluorescein. The side chains enable the substrate to enter the cell from the extracellular medium. Within the cell, the fluorescein generated by the antibody-catalyzed reaction can no longer pass readily through the membrane, causing a buildup of fluorescence within the cell. We describe the generation of this useful new substrate, test the ability of antibodies to generate fluorescence within living cytoplasm under various conditions, and demonstrate the applicability of the reaction by quantifying the transport of antibody-generated fluorescent tracers through gap junctions.

EXPERIMENTAL PROCEDURES

Fluorogenic Substrate Synthesis—Compounds are shown in Fig. 1. Methylallylmagnesium chloride solution (0.5 M in tetrahydrofuran (THF),[1] 50 ml, 25 mmol) at −78 °C was treated with acetone (1.9 ml, 25 mmol) in THF (20 ml). The mixture was warmed to −20 °C and treated with 3 ml of saturated aqueous ammonium chloride solution and 50 ml of ether. After 5 min at room temperature the mixture was dried (MgSO₄), filtered, and concentrated to give 2.64 g (23 mmol, 93%) of tertiary alcohol 1. 1H-NMR (250 MHz, CDCl₃): δ = 1.25 (s, 6H), 1.87 (s, 3H), 2.20 (s, 2H), 4.75 (s, 1H), 4.94 ppm (s, 1H).

Alcohol 1 (2.5 g, 21.93 mmol) in dry THF (10 ml) was added dropwise to a suspension of KH (3.77 g of a 35% suspension in mineral oil, 33 mmol, prewashed with dry hexanes) in dry THF (50 ml) at 0 °C under an argon atmosphere. The mixture was warmed to room temperature over 1.5 h and transferred dropwise via transfer needle to a solution of di-2-pyridyl carbonate (7.11 g, 33 mmol) in dry THF (50 ml). After 1 h the mixture was carefully treated with saturated aqueous ammonium chloride solution and 50 ml of ether. The organic layer was washed with saturated aqueous NaHCO₃, dried (MgSO₄), filtered, and concentrated to give 2.4 g (10.2 mmol, 47%) of the mixed carbonate 2 after column chromatography (hexanes/ethyl acetate 6:1). 1H-NMR (250 MHz, CDCl₃): δ = 1.63 (s, 6H), 1.92 (s, 3H), 2.64 (s, 2H), 4.84 (s, 1H), 5.00 (s, 1H), 7.20 (m, 1H), 7.30 (m, 1H), 7.85 (m, 1H), 8.46 ppm (m, 1H).

Carbonate 2 (2 g, 8.52 mmol) in dry CH₂Cl₂ was added dropwise to N,N′-dimethyl-ethane-1,2-diamine (9.2 ml, 85.2 mmol) in 60 ml of dry CH₂Cl₂. After 3 h at room temperature the mixture was concentrated and partitioned between ethyl acetate and brine, and the organic layer was dried (MgSO₄), filtered, and concentrated to give 1.94 g (8.52 mmol, >99%) of amine 3. 1H-NMR (250 MHz, CDCl₃): δ = 1.50 (s, 6H), 1.82 (s, 3H), 2.45 (s, 3H), 2.57 (s, 2H), 2.75 (broad singlet, 2H), 2.85 (s, 3H), 3.20 (sbr, 2H), 4.75 (s, 1H), 4.86 ppm (s, 1H)

Amine 3 (912 mg, 4 mmol) and triethylamine (614 µl, 4.4 mmol) in dry THF (10 ml) was treated with ZCl (623 µl, 4.4 mmol) at 0 °C. The mixture was warmed to room temperature, concentrated, and purified by column chromatography (ethyl acetate/hexanes 6:1/2:1) to give carbamate 4 (1.432 g, 3.96 mmol, 99%). High resolution mass spectroscopy calculated for $C_{20}H_{30}N_2O_4$-H^+: 363.2284, observed 363.2279.

Carbamate 4 (1.4 g, 3.87 mmol) in 15 ml of acetone was treated with OsO₄ (2.5% in *tert*-butanol, 500 µl) and 4-methylmorpholine (50% in

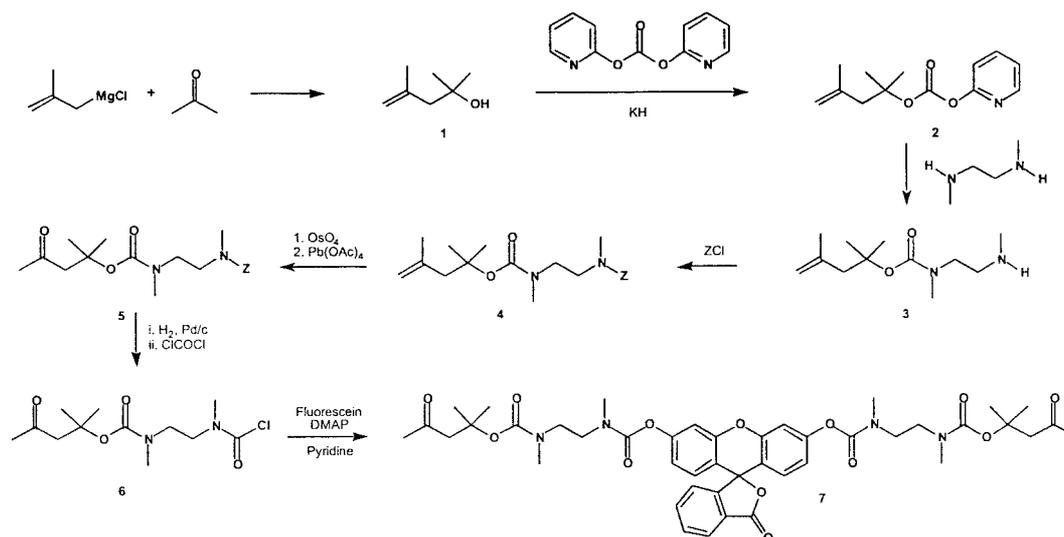[1] The abbreviations used are: THF, tetrahydrofuran; CHO, Chinese hamster ovary.

FIG. 1. **Synthesis of the fluorogenic substrate.** Compound **7** is the fluorogenic substrate used throughout the studies reported here. It readily passes through cell membranes into cells. The antibody-catalyzed conversion to fluorescein within cells removes the hydrophobic side chains, greatly reducing diffusion back through the membrane into the extracellular medium.

H_2O, 1.8 ml) and stirred for 2–3 h (TLC). The mixture was worked up with ethyl acetate/saturated aqueous $Na_2S_2O_5$ solution, the organic layers were dried ($MgSO_4$), filtered and concentrated, dissolved in 50 ml of dry CH_2Cl_2, and treated with $Pb(OAc)_4$ (4 mmol) at room temperature for 5 min. The mixture was treated with solid K_2CO_3, stirred for another 5 min, filtered over SiO_2, and washed with saturated aqueous NH_4Cl solution. The organic layer was dried ($MgSO_4$), filtered, and concentrated to give 1.25 g (3.44 mmol, 89%) of the ketone **5** after column chromatography (hexanes/ethyl acetate 3:1). 1H-NMR (250 MHz, $CDCl_3$): δ = 1.49 (s, 6H), 2.11 (s, 3H), 2.75–3.39 (m, 12H), 5.11 (s, 2H), 7.31 ppm (m, 5H)

Ketone **5** (200 mg, 0.55 mmol) in 8 ml of MeOH was hydrogenated with a catalytic amount of Pd/C. After filtration over celite, concentration, and reuptake in 5 ml of THF, triethylamine (160 μl, 1.1 mmol) was added, and this mixture was added to a solution of phosgene (570 ml, 1.1 mmol, 1.93 M in toluene) in 5 ml of dry THF at 0 °C. The mixture was worked up with saturated aqueous $NaHCO_3$ and ether. The organic layers were dried, filtered, and concentrated to give 145 mg (0.495 mmol, 90%) of chloride **6**. High resolution mass spectroscopy calculated for $C_{12}H_{21}ClN_2O_4$-Na^+: 315.1089, observed 315.1080.

Fluorescein (42 mg, 0.125 mmol) and carbamoyl chloride **6** (73 mg, 0.25 mmol in 500 ml of dry THF) in 1 ml of dry pyridine was treated with a catalytic amount of dimethylaminopyridine and stirred for 5 days at room temperature. After work up with ethyl acetate and saturated aqueous NH_4Cl, the mixture was purified by column chromatography (hexanes/ethyl acetate 1:2) to give carbamate **7** (13 mg, 0.0154 mmol, 12%). High resolution mass spectroscopy calculated for $C_{44}H_{52}N_4O_{13}$-Cs^+: 977.2585, observed 977.2602.

Determining the Kinetics of the Antibody-catalyzed Retro-Michael Reaction—A stock solution of fluorogenic carbamate **7** (10 mM in acetonitrile) was diluted with phosphate-buffered saline, pH 7.4, to the required concentrations and then treated with antibody 38C2 (67 μM, in phosphate-buffered saline, pH 7.4) purchased from Aldrich (Milwaukee, WI) to give a final antibody concentration of 1.47 μM regarding binding sites. To obtain kinetic data, the reactions were followed with a fluorescence plate-reader by monitoring at λ_{abs} = 485 nm and λ_{em} = 520 nm. The data was analyzed by using the program GraFit purchased from Erithacus Software Ltd. (Surrey, United Kingdom).

Cell Lines and Tissue Culture—Hybridoma cells expressing aldolase antibody 38C2 were kindly provided by Dr. Diane Kubitz. OKT4 cells, used as control hybridomas not expressing catalytic antibodies, were purchased from the ATCC (Manassas, VA). Both cell lines were grown in RPMI medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Gemini Bio-Products; Woodland, CA). CHO cells, also obtained from the ATCC, were grown in Ham's F-12 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Live Cell Experiments and Microscopy—Hybridoma cells expressing aldolase antibody 38C2 and OKT4 hybridoma cells were incubated with

fluorogenic substrate at a concentration of 20 μM for 8 h at 37 °C and 5% CO_2. Prior to the experiment, cells were washed and media was changed to phenol-free RPMI with 10% fetal bovine serum. CHO cells were grown in Ham's F-12 medium plus 10% fetal bovine serum. These cells were coinjected with 10,000 molecular weight rhodamine-dextran (Molecular Probes; Eugene, OR) and aldolase antibody 38C2 (10 mg/ml), then incubated with the fluorogenic substrate (20 μM), and maintained at 37 °C and 5% CO_2 for 8 or 12 h prior to the experiment. Immediately prior to the experiment, cells were washed, and the media was changed to phosphate-buffered saline with 10% fetal bovine serum. For oleamide treatments, a stock of oleamide (Calbiochem; La Jolla, CA) in pure ethanol was added to the cell medium to 1:1000 dilution, producing a final concentration of 100 μM. Glass coverslips of cells were mounted in a sealed Dvorak Chamber (Nicholson Instruments; Gaithersburg, MD) held in a temperature-controlled stage (20/20 Technologies). Microscopy was performed using an Axiovert 100 TV microscope (Carl Zeiss Inc.; Thornwood, NY) modified with automated stage and filter wheels (LEP Ltd.; Hawthorne, NY) and a Zeiss 40× NA1.3 oil immersion objective with differential interference contrast optics. Fluorescence images were obtained with an exposure time of 0.5 s using a Photometrics PXL cooled CCD camera (Photometrics; Tucson, AZ). Images were taken using 2 × 2 binning as a 12 bit, 658 × 517-pixel array. Images were background-subtracted and analyzed for fluorescence intensity (9) using Inovision ISEE software (Inovision Co.; Raleigh, NC). Fluorescence signals produced by the aldolase antibody reaction and rhodamine-dextran were imaged using HQ filter sets from the Chromatech Co. Fluorescence images were contrast-stretched and sharpened for clarity of display using Inovision ISEE and Adobe Photoshop software.

Gap Junction Dye Transfer Assay—This procedure was performed as previously described (10). These cells have been previously shown to contain gap junctions and connexin 43 (10–12). These results were confirmed for our cells through both Western blotting and immunofluorescence (data not shown).

RESULTS

Aldolase Antibody Efficiently Catalyzes Production of Fluorescein from a Fluorogenic Substrate—The catalytic antibody 38C2 was previously shown to efficiently catalyze retro-Michael reactions (8) and was therefore tested for its ability to convert a cell-permeable, fluorogenic substrate into fluorescein, a bright fluorophore with valuable properties for detection within living cells. The nonfluorescent fluorogenic substrate, compound **7** in Fig. 1, was synthesized from fluorescein and linker 2. Hydrophobic side chains conferred membrane permeability without completely eliminating water solubility. As shown in Fig. 2, the aldolase antibody 38C2 catalyzed the retro-Michael reaction of **7** to give a cyclic urea derivative, CO_2,
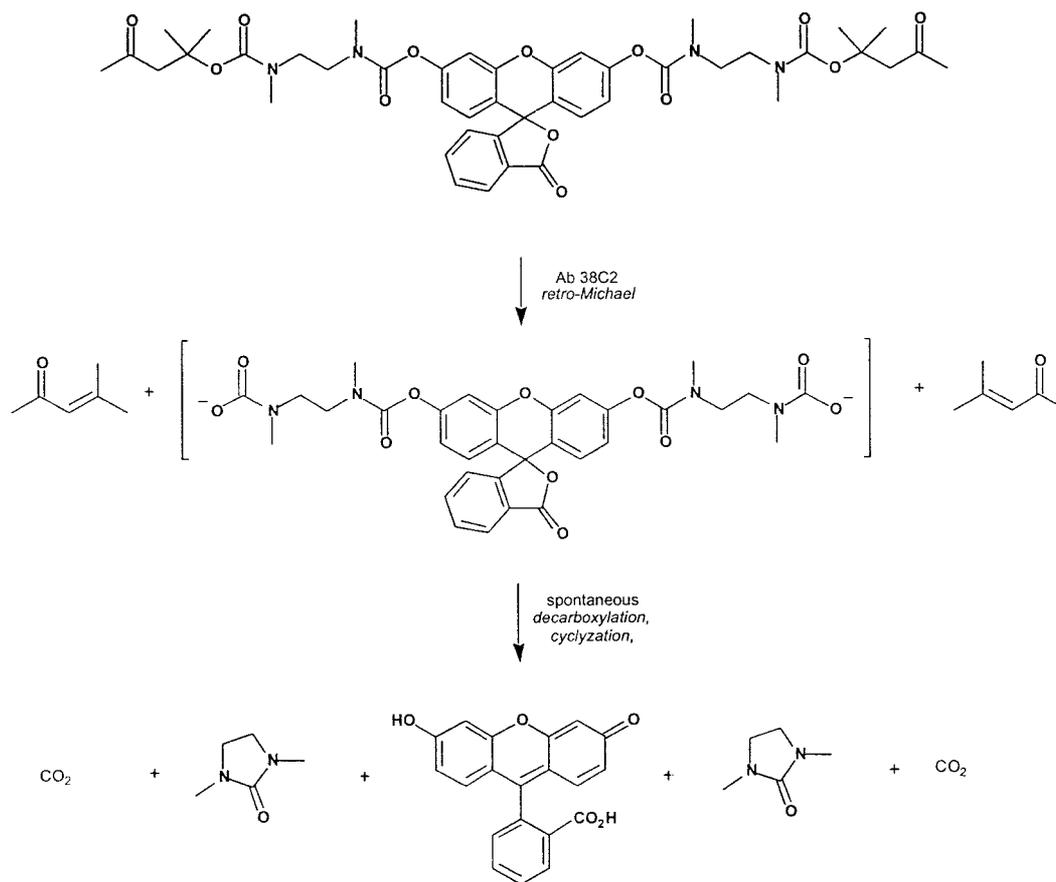
FIG. 2. **Antibody-catalyzed conversion of fluorogenic substrate to fluorescein.** The antibody catalyzes a retro-Michael reaction leading to an intermediate with free carboxylates that can spontaneously form fluorescein.

and mesitoxylide, which spontaneously rearranged to produce fluorescein. The k_{cat} of the antibody-catalyzed step was ~1.8 × 10^{-5} s^{-1} (K_m = 7 μM), and the catalytic proficiency (k_{cat}/ $k_{uncat}K_m$) was 1.4 × 10^{10} M^{-1}.

Aldolase Antibody Generates Fluorescent Dye within Living Cells—We examined whether the reaction between antibody and substrate could occur within the complex environment inside living cells and whether the amount of fluorescence generated was sufficient for practical application in biological assays. For this, we compared fluorescence generated when the fluorogenic substrate was loaded into hybridoma cells, which did or did not express the antibody. The substrate was incubated in the extracellular medium, cells were then washed with fresh medium free of substrate, and the fluorescence of individual cells was determined through quantitative image analysis. Results from more than 50 cells of each type are shown in Fig. 3. It was clear that the antibody reaction could proceed within living cells. The mean level of fluorescence was more than 12 times higher in the aldolase 38C2 antibody-expressing cells than in the control cells, so fluorescence due to the antibody reaction could be clearly differentiated from autofluorescence or from spontaneous reaction of the substrate to form fluorescent dye. These experiments demonstrated that the substrate could be taken up from the extracellular medium in sufficient concentrations to produce a good fluorescent signal. Finally, both cell lines showed no apparent toxic effects after the 8 h incubation.

It was important to determine whether fluorescence could be produced only by cells continuously generating antibody, such as hybridomas, or whether cells briefly expressing the antibody or loaded through introduction of extracellular antibody (*i.e.* through microinjection, electroporation, signal sequences, etc.)
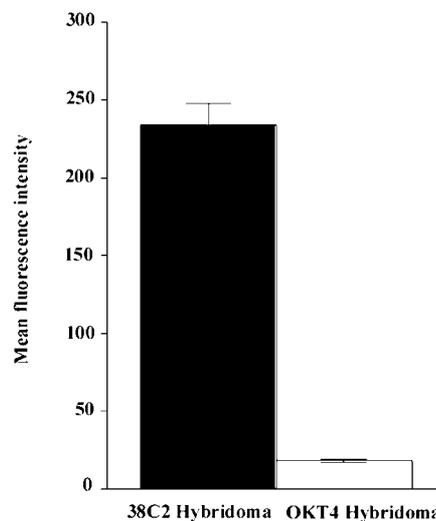

FIG. 3. **Fluorescein generated by intracellular antibody catalysis.** The fluorogenic substrate was incubated with hybridoma cells expressing the catalytic antibody (*dark bar*) and with control hybridomas expressing an irrelevant antibody (*light bar*). The mean fluorescence of the two populations was determined, demonstrating that the antibody was capable of generating fluorescent dye within living cells. Means were derived from 56 cells expressing catalytic antibody and from 69 control cells.

might also be able to generate fluorescence. Only the hybridoma cells might be able to generate sufficient intracellular concentrations of antibody, or the half-life of the antibody might be so short that constant expression was required. We examined the ability of microinjected antibody to generate flu-
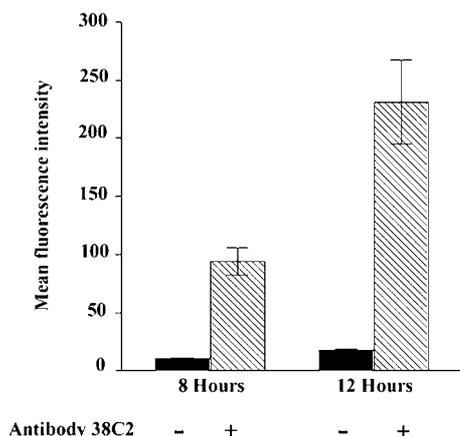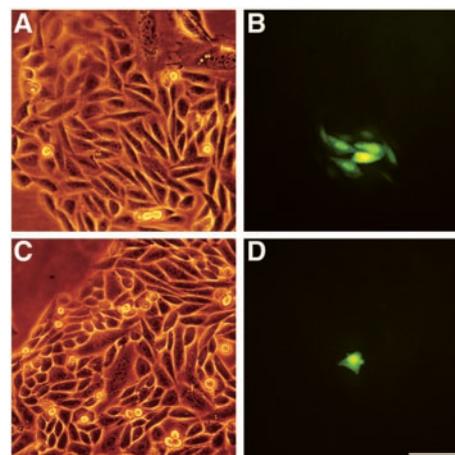
FIG. 4. **Cells injected with antibody efficiently generate fluorescence.** The mean fluorescence of CHO cells injected or not injected with antibody 38C2 was compared. After either 8 or 12 h of incubation with fluorogenic substrate, the injected cells produced a level of fluorescence readily differentiated from that of uninjected cells. Means were generated from three separate experiments using a total of 96, 172, 126, and 115 cells for the left to right bars, respectively.

FIG. 5. **Dye transfer through Gap junctions.** These representative images demonstrate that the CHO cells used for intracellular antibody reactions can undergo gap junction communication. Lucifer yellow dye injected into one cell was transferred into neighboring cells, as shown in *panels A* and *B*. When cells were treated with 50 μM oleamide for 30 min, transfer from the injected cell was blocked, as shown in *panels C* and *D*.
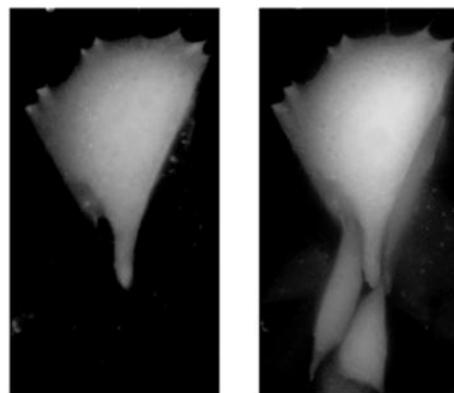
FIG. 6. **Gap junction communication.** Rhodamine fluorescence (*left panel*) and fluorescein fluorescence (*right panel*) is shown for the same group of cells. The large cell visible in the rhodamine channel was injected with a mixture of catalytic antibody and a 10,000-kDa rhodamine-dextran that cannot pass through gap junctions. The fluorescein image shows that the product of the antibody-catalyzed reaction passed through gap junctions into the smaller cells adjoining the injected cells.

orescent dye. For these experiments, CHO cells, a cell line commonly used for a wide range of biological experiments, were microinjected with the catalytic antibody, and the cells were then incubated with extracellular substrate for 8 or 12 h. Fig. 4 shows that the injected cells were also capable of generating bright, stable fluorescence easily detectable above background. Fluorescence was significantly increased at 12 h, indicating that the reaction was ongoing more than 8 h after injection and that the dye could accumulate within the cells. Here the mean fluorescence of the cells containing antibody was more than 13-fold greater than that of controls.

The intracellular antibody reaction would be more useful if the antibody not only generated a good mean fluorescence in a population of cells but also generated detectable fluorescence in a large percentage of cells injected. In the antibody injection experiments, fluorescence above background was seen in 87 and 93% of the injected cells at 8 and 12 h, respectively. (Cells were deemed fluorescent if they were more than one standard deviation brighter than the mean intensity of noninjected cells.)

Intracellular Aldolase Antibody Reaction Reveals Gap Junction Communication—Previous studies have demonstrated that CHO cells form gap junctions (10–12). Therefore, we examined whether the aldolase antibody reaction could be used to study gap junctions in these cells. By injecting the antibody into only one cell in a population of cells connected by gap junctions, the spread of fluorescence from the injected cell could be used to quantify gap junction activity. The transfer of lucifer yellow tracer dye through CHO cells is shown in Fig. 5. This finding demonstrates the functional activity of gap junctions in this cell line. Thus, these cells were suitable for testing the ability of the antibody reaction to quantify gap junction communication.

CHO cells were grown as a subconfluent monolayer on glass coverslips, such that cells were in clearly separate groups of ~5–10 adjoining cells. The catalytic antibody was mixed with a 10,000-kDa rhodamine-dextran. This dextran, too large to pass through gap junctions, served to mark the injected cells. The mixture of antibody and dextran was injected into only one cell within each group of adjoining cells. After 8 and 12 h, the number of noninjected cells that had become fluorescent was counted. Results are shown in Figs. 6 and 7. These experiments demonstrated that the substrate could be taken up from the extracellular medium in sufficient concentrations to produce a

uniform cytoplasmic fluorescent signal in the cells injected with the 38C2 aldolase antibody. Furthermore, cells surrounding the antibody-injected cells readily took up fluorescent substrate, while the fluorescent dextran marker remained in the injected cell only. Uptake was dependent on the time of incubation with substrate, consistent with the kinetics of fluorescent product formation seen in the antibody-injected cells (Fig. 4). Treatment with oleamide, a potent inhibitor of gap junction communication (13), greatly reduced the number of fluorescent noninjected cells. Together, these experiments demonstrated that the antibody reaction can be used to effectively trace gap junction communication.

DISCUSSION

The studies reported here demonstrated that a catalytic antibody, 38C2, generated bright and stable fluorescence within individual, living cells. This reaction was characterized to demonstrate that it is a practical tool with many potential applications. The novel substrate used here was delivered to the intracellular antibody simply by placing it in the extracellular medium. The level of fluorescence generated in cells was more than 10-fold greater than background fluorescence, enabling
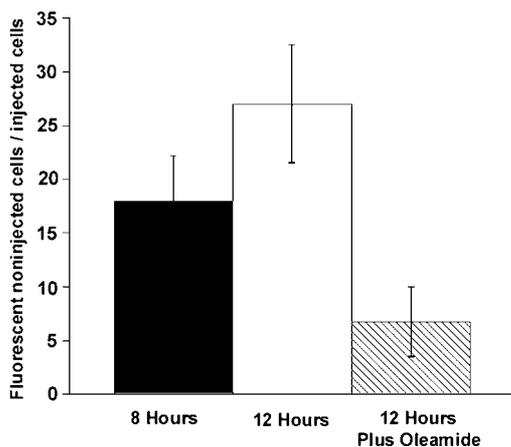
FIG. 7. **Intracellular antibody catalysis used to trace gap junction communication.** CHO cells were injected with a mixture of rhodamine-dextran marker and catalytic antibody, as shown in Fig. 7. Each *bar* shows the number of fluorescent noninjected cells divided by the number of injected, antibody-containing cells in each experiment after incubation with substrate for 8 h (*left*) or 12 h (*center*). Treatment with 100 μM oleamide greatly reduced the appearance of fluorescence in noninjected cells (*right*), indicating that dye formed by the antibody reaction was passing into noninjected cells through gap junctions. Division of fluorescent noninjected cells by injected cells normalizes for the different numbers of injected cells in each experiment. The results shown are the mean of three separate experiments ± S.E.

ready discrimination of antibody-containing cells. Fluorescence was generated both in cells constitutively expressing antibody and when antibody was loaded through microinjection. This demonstrated that constitutive expression was not required to achieve the required intracellular antibody concentrations or to overcome intracellular degradation of antibody.

For marking cells, intracellular antibody reactions have clear advantages over marker dyes used currently for long term tracing of cell fate. Constitutive expression of the antibody leads to continual regeneration of fluorescence, unlike the marker dyes that are diluted by cell division or interactions with other cells and decompose due to photobleaching. Perhaps most importantly, the antibody could be expressed transiently to generate fluorescence. Thus, it could be placed under the control of specific promoters to generate fluorescence only after activation of a regulated gene to correlate cell behavior with gene expression during development. Importantly, fluorescence was produced in greater than 85% of the cells injected, indicating that the reaction can be used reliably to determine which cells express antibody.

We used the antibody reaction to develop a new assay of gap junction activity. One cell within a population of adjoining cells was injected with antibody and the entire population was incubated with substrate. After the injected cell became fluorescent, dye was transferred into neighboring noninjected cells, which were in contact with the cell containing antibody. This

dye transfer was demonstrated to be mediated by gap junctions by blocking it with oleamide, a specific inhibitor of gap junction activity (13). There are currently no practical markers that can be used to quantify gap junction activity in living tissues or whole animals (14). We hope to use this new tool to investigate gap junction activity from specific metastatic cells during tumorigenesis, as the interactions of invading cells with surrounding tissue are thought to be mediated in part by gap junction transfer (15–17). There is also evidence that immune mediators are transferred via gap junctions to specific tissues (18–20).

This work has demonstrated the usefulness of catalytic antibody reactions within living cells. The reaction of antibody 38C2 with our novel substrate generated sufficiently bright and stable fluorescence for many potential applications. In the future, antibody-catalyzed reactions can be used not only to generate fluorescent dyes of multiple colors to simultaneously monitor different cells, but can generate other molecules that modulate cell activity. Appropriate substrates could generate inhibitors or other biologically active molecules. Alternately, the activity of native proteins could be modulated through binding of antibodies to alter conformation or accelerate natural reactions with natural modifying enzymes.

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