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# BINDING OF ANTI-TRIFLUOROACETYL ANTIBODIES TO ISOLATED HEPATOCYTES OBSERVED BY DIGITAL FLUORESCENCE MICROSCOPY*

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These experiments were designed to observe specific binding of fluorescein-conjugated FAB'2 secondary antibodies to epitopes on the surface of isolated hepatocytes. The hepatocytes were attached as monolayers on microscope cover slips and an antigenic adduct known to be formed during metabolism of halothane, -trifluoroacetyl-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, was exchanged into their surface. Then the monolayers of hepatocytes were incubated with primary rabbit antibodies specific for the trifluoroacetyl group. Each coverslip was mounted in a perfusion chamber on a fluorescence microscope and a set of digital fluorescence images was made. Then fluorescein-conjugated goat-anti-rabbit FAB'2 secondary antibodies were flowed over the monolayer, the perfusion chamber was washed with buffer, and a second set of digital fluorescence images was made. The difference of these two sets of images demonstrated intense fluorescene superimposed on the outline of the cells. This intense fluorescence was not observed in control experiments in which the primary antibodies were omitted. 

Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is oxidatively metabolized to trifluoroacetyl chloride and then to trifluoroacetic acid (1-3). Trifluoroacetyl chloride has been shown to modify hepatic proteins to produce antigens that can be detected by

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Abbreviations:
RSA; rabbit serum albumin. TFA-RSA; synthetic trifluoroacetylated rabbit serum albumin. Anti-TFA-RSA IgG; rabbit polyclonal IgG raised against TFA-RSA and affinity purified. FITC-goat-anti-rabbit FAB'2; fluorescein isothiocyanate-conjugated FAB'2 antibodies raised in goats against rabbit IgG. DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine. DOPC; 1,2-dioleoyl-sn-glycero-3-phosphocholine. TFA-DOPE; N-trifluoroacetyl-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine.

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antibodies to the trifluoroacetyl (TFA ) epitope (4,5). Several of these TFA-labeled proteins, which range from 22 to 110 kD, have been isolated and described in detail (6). It is thought that these antigens may be involved in the delayed onset form of halothane hepatitis, which is immune-mediated and often fatal (7). TFA-protein antigens have been detected *in vivo* in serum of halothane-exposed human patients (4,8) and in rats breathing room air (9) as well as *in vitro* in microsomes incubated with halothane in room air (6). It is becoming even more important to understand the role of antibody binding to TFA-adducts because trifluoroacetyl chloride is also formed by oxidative metabolism of the newer inhalational anesthetics isoflurane and desflurane as well as the new Freon substitutes HCFC-123, HCFC-124, and HFC-125 (10,11).

Using published methods, we have prepared and affinity purified (5,12) a polyclonal antibody in rabbits (anti-TFA-RSA IgG) that is specific for the synthetic antigen used in the immunizations, trifluoroacetylated rabbit serum albumin (TFA-RSA). In a study of possible cross-reactivity of these antibodies with antigenic phospholipids that could result from halothane metabolism, the N-trifluoroacetyl-adduct of phosphoethanolamine (TFA-DOPE) was prepared as described previously (13,14). It was shown that anti-TFA-RSA IgG antibodies bind TFA-DOPE when the latter antigenic phospholipids are in the surface of hexagonal phase micelles but not when they are in a host membrane of lamellar liposomes (12).

The lateral distribution of N-TFA-DOPE within the surface of the plasma membrane of hepatocytes is important for the interpretation of the latter results. Especially in the case of antigenic phospholipids introduced into a membrane by fusion, it is possible that the initial locally high concentrations at the fusion sites do not redistribute before the cross-reacting antibodies are bound. The present experiments were designed to observe the lateral distribution of antibody binding on hepatocytes in monolayer culture.

## MATERIALS AND METHODS

Both 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids, Inc. (Pelham, AL). Affi-Gel 10 affinity resins were from Bio-Rad (Richmond, CA). Fluorescein isothiocyanate-conjugated FAB'2 antibodies raised in goats against rabbit IgG (FITC-goat-anti-rabbit IgG) were from Sigma (St. Louis, MO).

An inverted Zeiss Axiomat (Zeiss, Oberkocken, Germany) was modified for epi-fluorescence microscopy. A 10 W Argon laser (Series 2000, Spectra Physics, Mountain View, CA) was used for excitation at 488 nm with 660 mW output. Neutral density filters were used for attenuation of the beam to a delivered intensity of 1.5

mW. The laser beam was passed through a telescope consisting of a short focus microscope lens (magnification X 10) and a collecting lens (focus = 10 mm). A quartz diffuser was mounted between these lenses to reduce formation of interference rings produced by the phase coherence of the laser. A dichroic mirror reflected the excitation beam through a Zeiss Planapo objective (X 100, 1.3 n.a., oil) to the sample. A part of the fluorescence emission was reflected back through the dichroic mirror and an interference filter (515-537 nm, Hugo Anders, Germany). This light was directed onto a photomultiplier (E.M.I., type 9862B/350, England) and a photon counting system (Ortec, Germany) to yield the integrated fluorescence intensity emitted from selected cells. Alternatively, digital fluorescence images were acquired with an intensified VIM camera (S2400/20, Hamamatsu Phototonics, Japan). This video signal was routed to a digital frame buffer (VTE Digital Video, Germany) where digital images of 512 by 512 pixels were stored in real time. The images were then transferred to a VAXstation 3200 (Digital Equipment, Corp., Maynard, MA). The 30 averaged background frames were subtracted from 30 averaged frames of hepatocytes that have been exposed to fluorescent antibodies. The resulting digital image was smoothed by averaging blocks of 8 by 8 pixels into single pixels to produce a 64 by 64 pixel surface plot on an Iris 4D/35 workstation (Silicon Graphics, Santa Clara, CA).

N-trifluoroacetylated rabbit serum albumin (TFA-RSA) was prepared as previously described (5,12). This antigen was injected into rabbits and also coupled to an Affi-Gel 10 (Biorad) affinity column. IgG antibodies specific for TFA-RSA were prepared by binding the crude IgG fraction to this affinity column and eluting the anti-TFA-RSA IgG antibodies with 2.5 M $MgCl_2$ (5,12). A purified sample of IgG antibodies from serum of the same rabbits before immunization (pre-immune-IgG) was prepared to use as a control for non-specific binding.

Hepatocytes were prepared from adolescent male Sprague Dawley rats (180-240 g). The rats were anesthetized with diethyl ether, and hepatocytes were isolated by in situ perfusion of the livers with collagenase, as previously described (15,16). The experimental animal protocol was approved by the committee of the Bezirksregierung Braunschweig. The 24.5 mm glass coverslips that fit into the perfusion chamber were coated with rat tail collagen (Boeringer Manheim, Germany) and attached temporarily to the center of 35 mm culture dishes with drops of paraffin. The coverslips were then pre-incubated for 3 h with 20% bovine calf serum before 1 ml aliquots containing approximately 1.5 x $10^6$ hepatocytes were added to the coverslips in the culture dishes. After a 90 min attachment period the hepatocyte monolayers were washed twice. Cells at this point were approximately 95% viable. The hepatocytes on the coverslips were incubated an additional 20 h and then washed again.

The TFA-DOPE was exchanged into the surface plasma membrane of the hepatocytes attached to the coverslips by preparing very small sonicated vesicles of the phospholipids and equilibrating them with the membranes (14,17). N-trifluoroacetyl-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (TFA-DOPE) was prepared as described previously (13) and purified by HPLC (14). Liposomes containing 50% by weight TFA-DOPE (75 $\mu$g) and 50% DOPC (75 $\mu$g) in 5 ml of HEPES buffer (140 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 10 mM HEPES, 3 mM glucose, pH 7.4) were prepared by sonication in a bath sonicator for 3 X 1 min at room temperature. Then 0.25 ml of these liposomes were added to a culture dish containing a 1 ml HEPES buffer over a monolayer of hepatocytes on a coverslip and the mixture was incubated for 45 min at 37 °C. The monolayers were then washed to remove liposomes that had not fused with the hepatocytes.

The cells were then incubated with rabbit anti-TFA-RSA IgG antibodies(10 $\mu$g) for 30 min at 37 °C, washed thoroughly, and the coverslips were mounted in the

perfusion chamber. A microscope field containing three contiguous hepatocytes surrounded by an area without debris was selected. Throughout the experiment the cells were constantly superfused with HEPES buffer at 100 µl/min and maintained at 37 °C. According to a previously written computer program the cells were rinsed with HEPES buffer for 3 min and over the next 2 min 30 digital image were made and averaged. The average of these images was a measure of autofluorescence from the hepatocytes and background fluorescence from the perfusion chamber. Then the cells were exposed to a 100 µl/min flow of FITC-conjugated goat-anti-rabbit FAB'2 antibodies (10 µg/ml) for 20 min. Fluorescence was monitored with a photomultiplier during the addition of the secondary antibody. The chamber was washed for 9 min with HEPES buffer. This time was sufficient to remove the unbound FITC-conjugated secondary antibodies, as shown by the photomultiplier trace (Figure 1) that quickly reached a steady state at a value higher that the control. Then at 34 min a second set of 30 digital images was made and averaged. The average of the first set of digital images was subtracted from the second.

The preceding experiment was repeated with the same result. Then duplicate control experiments were performed in identical steps except that no anti-TFA-RSA IgG antibodies were added to the first incubation with the coverslips.

## RESULTS AND DISCUSSION

The increase in fluorescence as FITC-conjugated FAB'2 secondary antibody was flowed over the monolayer was monitored with a photomultiplier (Figure 1). A constant upward inflection can be seen in the trace between 5 and 25 min and the level at 30 min is 1.1 X $10^4$ counts/sec compared to the initial value of 9 x $10^3$

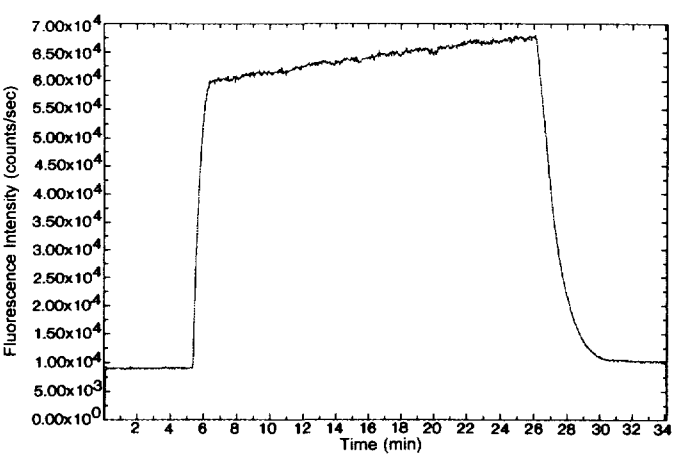


Figure 1. Integrated fluorescence intensity measured during the experiment. The fluorescence intensity emitted from the chamber was monitored with a photomultiplier and a photon counting system. The fluorescence intensity (counts/min) is shown during a 5 min wash, a 20 min superfusion with fluorescent antibodies, and a 9 min wash. Digital images where recorded at times 3-5 min and 34-36 min. It is seen that the intensity builds during the labeling period and returns to a level above baseline after the second wash.

counts/sec. In contrast, in the corresponding controls the trace was flat in the 5 to 25 min region and the initial and final levels were not different. After 20 min the unbound antibodies were washed away with buffer, and a second set of digital fluorescence images was made. The difference of the first and second set of images demonstrated intense fluorescence superimposed on the outline of the three cells (Figure 2). Duplicate control experiments, in which incubation of the hepatocytes with the primary anti-TFA-RSA IgG antibodies was omitted, showed only an even low level of fluorescence that is characteristic of NADH and NADPH autofluorescence in hepatocytes (18).

The subtracted digital fluorescence image in Figure 2 shows that anti-TFA-RSA IgG antibodies are able to bind to TFA-DOPE in the plasma membrane of hepatocytes. This result suggests that TFA-adducts of phospholipids could act as haptenic binding sites for anti-TFA antibodies and have a role in immune-mediated halothane hepatitis. In addition, the pattern of fluorescence shown in Figure 2 demonstrates that the distribution of secondary antibody binding sites on the surface of the hepatocytes is relatively uniform. Therefore, the technique of fusing liposomes into the plasma membrane of hepatocyte results in an even distribution of antigenic phospholipids within 45 min.

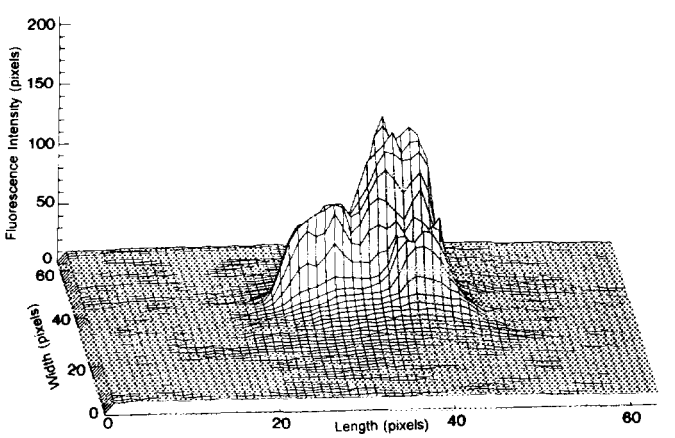


Figure 2. Surface plot of fluorescence emitted from three hepatocytes. Isolated hepatocytes were grown as monolayers on microscope coverslips, TFA-DOPE was exchanged into their surfaces, they were incubated with the primary anti-TFA-RSA IgG antibodies, and then washed. A coverslip was mounted in a perfusion chamber on a fluorescence microscope and a set of digitized fluorescence images of three cells was made. Then fluorescein-conjugated FAB'2 secondary antibodies were flowed over the monolayer, the unbound antibodies were washed away with buffer, and a second set of digital fluorescence images was made. This surface plot is the difference of the first and second set of images. The vertical axis is fluorescence intensity and the outline of the three hepatocytes is seen in the x and y dimensions.

It should be emphasized that the strong binding of anti-TFA-RSA IgG antibodies to TFA-DOPE when it is fused into the surface of hepatocytes is somewhat unexpected. A series of previous studies has shown that cross-reactive antibodies raised against antigens coupled to protein carriers can bind to antigenic phospholipids (19,20). In general, these studies were carried out in lamellar phase liposomes or supported bilayers (21-23). However, antibody binding to haptenic phospholipids imbedded in lamellar liposomes requires a spacer of several carbon atoms between the hapten and the phospholipid headgroup (22,24,25). As a result, it was expected that antibodies would not bind to TFA-DOPE in the surface of lamellar liposomes because it lacks an adequate spacer.

It has been shown previously that anti-TFA-RSA IgG antibodies bind TFA-DOPE when the latter antigenic phospholipids are in the surface of hexagonal phase micelles, but not lamellar liposomes (12). This finding was supported by recent studies that have demonstrated that the antigenicity of phospholipids in hexagonal phase micelles is greatly enhanced in comparison to lamellar phase liposomes (26-28). For example, lupus anticoagulants, a subclass of anti-phospholipid antibodies, distinguish between lamellar and hexagonal phase phospholipids and bind only to the latter phase (29). Evidence for the existence of inverted micelles within hepatocyte microsomal membranes has been provided by [$^{31}$P]-NMR spectroscopy (30). Although inverted micelles are spherical, whereas hexagonal phase micelles are a bundle of extended tubes, they share a similar ability to disrupt the orderly packing of biological membranes.

Our present result that anti-TFA-RSA IgG antibodies bind to TFA-DOPE in the surface of hepatocytes is supported by the earlier flow cytometry result that anti-N-ethyl-RSA IgG antibodies bind to N-ethyl-DOPE after it was fused into the plasma membrane of hepatocytes (14). Both of these results are consistent with the suggestion that there are areas on the plasma membrane of hepatocytes in which haptenic phospholipids can be recognized more easily than in lamellar liposomes.

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