micronuclei that are the result of broken chromosomes (*17*). That the present results are not attributable to perturbations in nucleotide pools, however, can be deduced from the fact that only the radioactive form of thymidine led to the effect, even though both radioactive and nonradioactive thymidine were present at the same concentration. Furthermore, the labeling patterns of the chromosomes in the autoradiographs prepared from cells cultured in the presence of [^{3}H]dThd indicated that the [^{3}H]dThd was completely used in the first cell cycle, so that in all cases the second cycle was passed in the absence of exogenous thymidine, and all of the cells should have been subjected to the same imbalances. Thus, the phenomenon is most likely caused by the radiation from the tritium.

The major effect observed in this study [see also (*18*)] was that radiation from incorporated isotopes seems to trigger a response that is analogous to the adaptive response to alkylating agents reported by Samson and Schwartz (*4*). In their experiments, cells chronically exposed to small doses of an alkylating agent were resistant to subsequent exposures to large acute doses of the same chemical. The response was not related to differences in cell cycling. In the present experiments, in which cells were given 150 rad of x-rays after exposure to chronic radiation from an incorporated radioisotope, the response was similar to the adaptive response, but now it occurred for ionizing radiation–induced chromatid aberrations in human lymphocytes. The results are consistent with the concept that exposure to low levels of chronic radiation can trigger or induce increased repair of radiation-induced chromosome breaks.

This putative radiation-stimulated repair is reminiscent of the SOS repair frequently reported to be induced in bacterial systems (*19*), although SOS repair leads to more, not fewer, mutations. Furthermore, because hydrogen peroxide and ionizing radiation yield similar reactive species, Demple and Halbrook (*20*) tested the effect of prior treatment with H_2O_2 on the resistance of *Escherichia coli* to x-rays. They concluded that peroxide induces the repair of lesions induced by peroxide itself and by ionizing radiation. In *Drosophila melanogaster*, x-rays themselves have been reported to induce DNA repair and thus reduce mitotic recombination (*21*).

Perhaps of more specific interest are reports that the activity of the enzyme poly(ADP-ribose) polymerase (ADP, adenosine diphosphate), which has been involved in repair, is increased by DNA strand breaks of the type produced by chronic radiation from incorporated [^{3}H]dThd (*22*). However, whether the induction of this enzyme is related to the adaptive response observed in the present experiments is unknown.

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Identification of an Erythrocyte Component Carrying the Duffy Blood Group Fy^a Antigen

Abstract. *The erythrocyte component carrying the Duffy blood group antigen Fy^a has been identified as a 35- to 43-kilodalton protein. The protein is degraded by proteases, chymotrypsin, and Pronase, which destroy its antigenicity on intact erythrocytes. Its unusual property of aggregating on being boiled in 5 percent sodium dodecyl sulfate with 5 percent 2-mercaptoethanol distinguishes it from other erythrocyte membrane proteins described to date.*

The Duffy blood group system consists of four major phenotypes, Fy(a+ b−), Fy(a− b+), Fy(a+ b+), and Fy(a− b−) (*1*). The system is defined by two antisera, anti-Fy^a and anti-Fy^b; Duffy negative erythrocytes, Fy(a− b−), are not agglutinated by either antiserum. With rare exceptions, the Duffy negative phenotype occurs only in blacks. Blacks with Duffy negative erythrocytes cannot be infected by the human malaria parasite *Plasmodium vivax* (*2*). Duffy negative human erythrocytes are resistant to invasion in vitro by a monkey malaria parasite, *Plasmodium knowlesi*, that can invade Duffy positive human erythrocytes (*3*). Anti-Fy^a and anti-Fy^b sera block invasion of Fy(a+ b−) and Fy(a− b+) erythrocytes, respectively (*3, 4*). These observations suggest that molecules containing the Duffy determinants are involved in the invasion process by these malarias.

Data on the molecular nature of the Duffy antigens are limited and inconclusive (*5*). In the present study we combined the high resolution of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with protein blotting or electroelution in order to identify an erythrocyte membrane component that carries Fy^a determinants. The component, which has an apparent molecular weight of 35,000 to 43,000, appears to be different from previously described erythrocyte components.

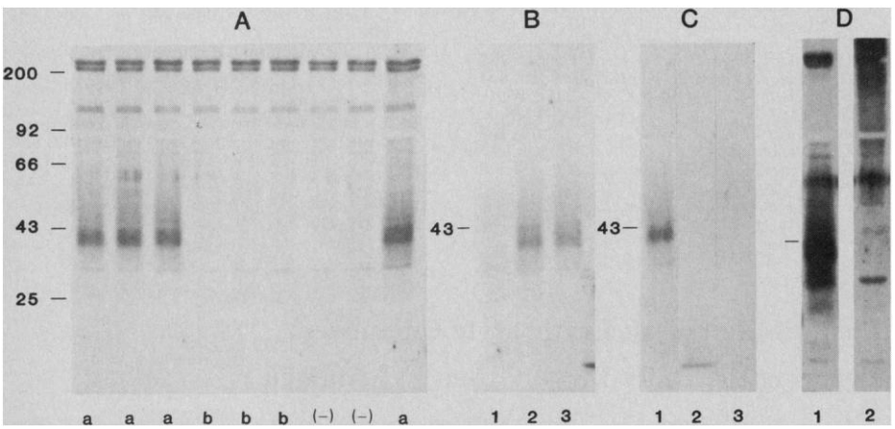
Erythrocyte ghosts were prepared from Fy(a+ b−), Fy(a− b+), and Fy(a− b−) erythrocytes and extracted in 5 percent (weight to volume) SDS and 5 percent (by volume) 2-mercaptoethanol. Components of each extract were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose paper, which was then reacted with human serum containing anti-Fy^a specificity (*6, 7*). Iodine-125–labeled protein A (Amersham) was used as a probe to locate antigen-antibody complexes on the paper.

Anti-Fy^a reacted specifically with an erythrocyte membrane component mi-

grating as a broadband between actin (band 5) and glyceraldehyde-3-phosphate dehydrogenase (band 6) and having an apparent molecular weight of 35,000 to 43,000 (Fig. 1A). Some reactivity was also visible extending upward to around 66 kilodaltons (kD), but this appeared weak in comparison to the major band at 35 to 43 kD. This band was seen only when anti-Fy^a was reacted with blots prepared from Fy(a+ b−) cells and not with blots prepared from Fy(a− b+) or Fy(a− b−) cells (Fig. 1A). These findings were consistent for the four Fy(a+ b−) donors, three Fy(a− b+) donors, and two Fy(a− b−) donors tested. Reactivity of anti-Fy^a with spectrin and some additional components was considered to be nonspecific since it was seen with Fy(a− b+) and Fy(a− b−) as well as Fy(a+ b−) cells. When normal human serum and anti-Fy^b were reacted with blots of Fy^a ghosts, no reactivity was seen other than the same nonspecific bands. Similar experiments performed with Fy(a− b+) cells and anti-Fy^b showed no specific reactivity.

Treatment of intact erythrocytes with chymotrypsin at a concentration of 1 mg/ml is known to remove Fy^a, whereas trypsin treatment does not (*3*). Therefore Fy(a+ b−) erythrocytes were analyzed after treatment with these enzymes. As expected, chymotrypsin-treated Fy(a+ b−) erythrocytes were no longer agglutinated by anti-Fy^a, and trypsin-treated erythrocytes were agglutinated normally. Correspondingly, analysis of these same erythrocytes showed a loss of the 35- to 43-kD band after treatment with chymotrypsin, whereas trypsin had no effect (Fig. 1B). These findings support the conclusion that the Fy^a antigen is carried on the 35- to 43-kD component on the erythrocyte surface.

Two additional experiments provided direct evidence that anti-Fy^a bound in the 35- to 43-kD region of the protein blot and that the Fy^a antigen was in this region of the gel. First, anti-Fy^a was affinity-purified from human serum con-



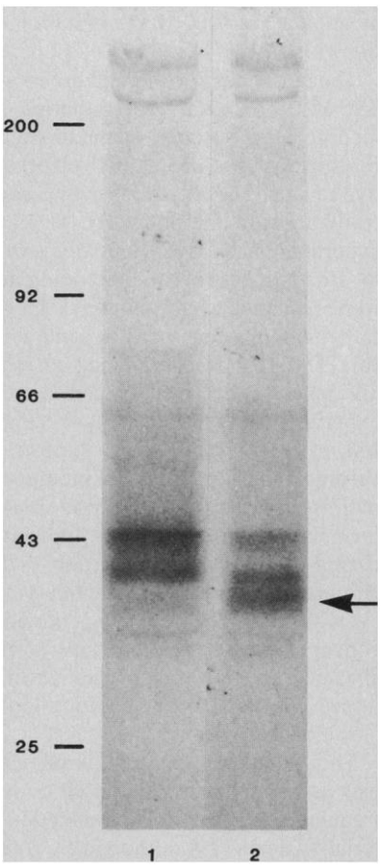


Fig. 1 (left). Immunoblots of electrophoretically separated erythrocyte membrane components reacted with anti-Fy^a and then ^{125}I-labeled protein A. (A) Erythrocyte ghosts were prepared by hypotonic lysis with 40 volumes of 5 m*M* sodium phosphate (*p*H 8.0) containing 1 m*M* phenylmethylsulfonyl fluoride (PMSF; Sigma) for 30 minutes at 4°C. After several washes in the same solution at 4°C, the membranes were extracted in four volumes of 5 percent (weight to volume) SDS and 5 percent (by volume) 2-mercaptoethanol. Samples were subjected to SDS-PAGE with 3 percent acrylamide in the stacking gel and 7.5 percent acrylamide in the resolving gel by using the discontinuous buffer system of Laemmli (*9*). Prestained standards (BRL) were used as molecular weight references. Electroblotting was performed in tris-glycine (25 m*M* tris base and 192 m*M* glycine) containing 20 percent (by volume) methanol by using a Trans-Blot Cell (Bio-Rad) overnight (12 to 16 hours) at 4°C and 30 V. The nitrocellulose (Schleicher & Schuell BA 85) was quenched in phosphate-buffered saline (PBS) (*p*H 7.4) containing 0.3 percent Tween 20 (Sigma) for 1 hour at room temperature with gentle agitation. After two 30-minute washes in 0.05 percent Tween 20 in PBS (washing buffer), the paper was reacted with anti-Fy^a (agglutination titer, 1:526), diluted 1:100 in washing buffer, and gently agitated overnight at 4°C. The paper was given four 20-minute washes in washing buffer and then reacted with ^{125}I-labeled protein A (2.5×10^6 count/min) diluted in washing buffer for 1 hour at room temperature. After multiple washes the paper was placed on Gelbond (Bio Products) and dried overnight at room temperature. Autoradiography was done with Kodak XAR-5 film. The notations *a*, *b*, and (−) at the bottom refer to Fy(a+ b−), Fy(a− b+), and Fy(a− b−) erythrocytes, respectively. The numbers at the left are the molecular weight standards (kilodaltons): myosin (200), phosphorylase B (92), bovine serum albumin (66), ovalbumin (43), and α-chymotrypsinogen (25). (B) Enzymatic treatment of Fy(a+ b−) erythrocytes (2×10^8 cells per milliliter) was done with α-chymotrypsin (1 mg/ml) (lane 1), trypsin and L-1-tosylamide-2-phenylethyl chloromethyl ketone (1 mg/ml; Worthington) (lane 2), or no enzyme (lane 3) for 2 hours at 37°C in RPMI 1640 medium (Gibco). After two washes in 100 volumes of RPMI 1640, erythrocytes were incubated for 15 minutes at room temperature with the appropriate protease inhibitor, 1 m*M* PMSF (Sigma), or soybean trypsin inhibitor (10 mg/ml; Sigma), and then washed again in 50 volumes of RPMI 1640. Ghosts were prepared, extracted, electrophoresed, and electroblotted as described above. (C) Extracts of Fy(a+ b−) erythrocytes in 1 percent sodium deoxycholate (Sigma) and 10 m*M* Hepes (Sigma) (*p*H 8) were treated with α-chymotrypsin (1 mg/ml; Worthington) (lane 2), Pronase (1 mg/ml; Calbiochem) (lane 3), or no enzyme (lane 1) for 3 hours at room temperature. Extracts were centrifuged at 80,000*g* for 30 minutes at room temperature and the supernatants were added to an equal volume of 10 percent (weight to volume) SDS and 10 percent (by volume) 2-mercaptoethanol prior to SDS-PAGE. (D) Fy(a+ b−) erythrocyte ghosts extracted in 5 percent SDS and 5 percent 2-mercaptoethanol were either left at room temperature (lane 1) or boiled for 3 minutes (lane 2) before electrophoresis and electroblotting. Note aggregates of Fy^a antigen at the top of the gel after boiling (lane 2). In the experiments represented in (B) through (D), Fy(a− b+) erythrocytes processed in parallel as a control showed no reactivity with anti-Fy^a. Fig. 2 (right). Comparison of Fy(a+ b−) erythrocyte ghosts treated with *Vibrio cholera* neuraminidase (Calbiochem) (lane 2) with the pattern of untreated ghosts (lane 1). One-hundred microliters of a ghost suspension (1.0 mg of protein per milliliter) was incubated with neuraminidase (0.3 unit/ml) for 45 minutes at 37°C in the presence of 0.2 m*M* *p*-tosyl-L-lysine chloromethyl ketone, 0.2 m*M* L-1-tosylamide-2-phenylethyl chloromethyl ketone, pepstatin (10 μg/ml), 2 m*M* PMSF (all from Sigma), and leupeptin (0.25 μg/ml; Boehringer Mannheim). Ghosts were extracted in an equal volume of 10 percent (weight to volume) SDS and 10 percent (by volume) 2-mercaptoethanol and reacted with anti-Fy^a after protein blotting. Similar results were obtained at neuraminidase concentrations of 0.1 and 0.05 unit/ml.

taining anti-Fy^a by using the 35- to 43-kD region of an SDS-polyacrylamide gel blotted on diazotized paper. Protein blotting of Fy(a+ b−) ghosts after SDS-PAGE was performed with nitrocellulose paper replaced by aminobenzyloxymethyl-modified paper (ABM, Schleicher & Schuell), which binds proteins covalently. A strip of paper in the 35- to 43-kD region and a control strip in the region of band 3 were cut out, incubated with anti-Fy^a serum, and washed extensively. Bound antibodies were acid-eluted and tested by agglutination against Fy(a+ b−) and Fy(a− b+) erythrocytes. Antibodies eluted from the strips agglutinated Fy(a+ b−) cells at a titer of 1/32 but not Fy(a− b+) cells. Eluate from the band 3 strip did not agglutinate erythrocytes of either Duffy type.

In the second experiment, molecules in the 35- to 43-kD region of the gel were electroeluted and shown to inhibit agglutination of Fy(a+ b−) erythrocytes by anti-Fy^a (Table 1). This eluate had no effect on agglutination of Fy(a− b+) cells by anti-Fy^b. Molecules from other regions of the gel had little or no effect on agglutination of Fy(a+ b−) cells by anti-Fy^a.

The fact that Fy^a activity was removed by treatment of Fy(a+ b−) erythrocytes with chymotrypsin suggested that the molecule bearing the Fy^a antigen is a protein. In further experiments Fy(a+ b−) erythrocyte ghosts were extracted in 1 percent Triton X-100 or 1 percent sodium deoxycholate. These extracts were treated for 3 hours at room temperature with chymotrypsin (1 mg/ml) or Pronase (1 mg/ml) and analyzed by SDS-PAGE and protein blotting. Both enzymes degraded the 35- to 43-kD component (Fig. 1C). The band seen at the bottom of the gel near the dye front after chymotrypsin treatment of Fy(a+ b−) extracts was also seen after chymotrypsin treatment of Fy(a− b+) extracts and is probably nonspecific rather than a fragment containing Fy^a determinants.

To determine whether the 35- to 43-kD component contains sialic acid, Fy^a erythrocyte ghosts were treated with *Vibrio cholera* neuraminidase before SDS-PAGE, protein blotting, and reaction with anti-Fy^a. After neuraminidase treatment a band of increased intensity appeared at 31 kD; areas above showed decreased intensity (Fig. 2). This altered mobility indicates that some Fy^a reactive molecules contain sialic acid. Neuraminidase-treated Fy^b ghosts were analyzed in parallel and showed no reactivity other than the nonspecific bands observed previously. The finding that neuraminidase treatment of Fy^a ghosts did not remove Fy^a reactivity indicates that sialic acid is not required for Fy^a antigenic activity. This is consistent with the observation that treatment of Fy^a (or Fy^b) erythrocytes with neuraminidase does not diminish Fy^a (or Fy^b) activity when measured by standard agglutination assays (*8*).

A peculiar characteristic of the 35- to 43-kD component was that, on boiling in 5 percent (weight to volume) SDS with 5 percent (by volume) 2-mercaptoethanol, anti-Fy^a no longer bound to the 35- to 43-kD region of the gel blot and instead bound to a region of the blot at the top of the gel (Fig. 1D). This reactivity of anti-Fy^a at the top of the blot was specific in that it did not occur with Fy^b ghosts processed in parallel. No change in the appearance of the band occurred when cells were extracted and boiled in 5 percent (weight to volume) SDS without 2-mercaptoethanol. This suggests that disruption of intramolecular disulfide bonds is a necessary step for the formation of aggregates. Whether the aggregates result from intermolecular disulfide bonding or other molecular interactions is not known. However, the unusual property of aggregating on boiling in SDS and 2-mercaptoethanol distinguishes the 35- to 43-kD component from other erythrocyte components, such as monomeric glycophorin A or dimeric glycophorin B, that migrate in this region.

Since the component is sensitive to degradation by proteases, both when intact erythrocytes and sodium deoxycholate extracts of erythrocyte ghosts are treated with chymotrypsin or Pronase, we conclude that this component carrying the Fy^a antigen is a protein. Since neuraminidase alters the component's mobility, we believe it to be a glycoprotein. The broadness of the 35- to 43-kD band may be due to variable glycosylation. Its peculiar property of aggregating upon boiling in SDS and 2-mercaptoethanol distinguishes it from other erythrocyte membrane proteins described to date. The assay we used should be useful in efforts to purify the protein. Once purified, its function with respect to the erythrocyte and its role as a possible receptor for *P. knowlesi* and *P. vivax* can be investigated.

Table 1. Inhibition of anti-Fy^a-induced agglutination of Fy(a+ b−) erythrocytes by eluates from the 30- to 43-kD region of SDS-polyacrylamide gel. One milliliter of extract of Fy(a+ b−) cells was applied to each of two 7.5 percent polyacrylamide gels, with prestained standards (BRL) being used to determine molecular weights. Using the 43-kD marker as a starting point, we obtained 1.5-cm-wide slices. Molecules from corresponding slices of each gel were electroeluted into a concentrating chamber (1750 Concentrator, Instrumentation Specialities) (*10*). Samples were electroeluted into 10 m*M* tris acetate (*p*H 8.6) containing 0.1 percent deoxycholate. To remove the deoxycholate, samples were dialyzed for 4 days against a total of 4 liters of 150 m*M* NaCl and 10 m*M* tris (*p*H 8.2) and then for 1 day against 1 liter of 140 m*M* NaCl, 2.7 m*M* KCl, and 10 m*M* $NaPO_4$ (*p*H 7.4). Serial dilutions of anti-Fy^a were made and each dilution was incubated for 30 minutes at room temperature with an equal volume of dialyzed eluate from the gel slices to be tested. Agglutination of Fy(a+ b−) erythrocytes by each of these dilutions was then determined by standard blood bank methods with dialysis buffer as a control.

Molecular weight of gel slice (kilodaltons)	Agglutination titers after incubation of eluates from gel slices with anti-Fy^a			
	Experiment			
	1	2	3	4
Buffer	1:256	1:256	1:128	1:128
150 to 210			1:128	
110 to 150			1:128	
80 to 110	1:256	1:256	1:128	
55 to 80			1:128	1:64
43 to 55			1:64	1:64
30 to 43*	1:16	1:32	1:16	1:8
25 to 30				1:64
Dye front, 25				1:64

*Molecular weight region corresponding to the 35- to 43-kD Fy^a component identified by protein blotting. Note the marked reduction in agglutination titers caused by eluates from this region.

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