

# Intracellular Distribution and Degradation of Immunoglobulin G and Immunoglobulin G Fragments Injected into HeLa Cells

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**ABSTRACT** Intact rabbit immunoglobulin G molecules (IgGs) and their papain or pepsin fragments were radio-iodinated and injected into HeLa cells. Whole IgGs, Fab<sub>2</sub>, and Fc fragments were degraded with half-lives of 60–90 h, whereas half-lives of Fab fragments were 110 h. These results indicate that proteolytic cleavage in the hinge region of the IgG molecule is not the rate-limiting step in its intracellular degradation. The hingeless human myeloma protein, Mcg, was degraded at the same rate as bulk human IgG, providing further evidence that the proteolytically susceptible hinge region is not important for intracellular degradation of IgG molecules.

SDS acrylamide gel analysis of injected rabbit IgG molecules revealed that heavy and light chains were degraded at the same rate. Injected rabbit IgGs and rabbit IgG fragments were also examined on isoelectric focusing gels. Fab, Fab<sub>2</sub>, and Fc fragments were degraded without any correlation with respect to isoelectric point. Positively charged rabbit IgGs disappeared more rapidly than their negative counterparts, contrary to the trend reported for normal intracellular proteins. The isoelectric points of two mouse monoclonal antibodies were essentially unchanged after injection into HeLa cells, suggesting that the altered isoelectric profile observed for intact rabbit IgG resulted from degradation and not protein modification.

The intracellular distributions of IgG fragments and intact rabbit IgG molecules were determined by autoradiography of thin sections through injected cells. Intact IgG molecules were excluded from HeLa nuclei whereas both Fab and Fc fragments readily entered them. Thus, for some proteins, entry into the nuclear compartment is determined primarily by size.

Cells degrade cytosolic proteins at differing rates (3, 18, 19). It was shown almost thirty years ago that this selective degradation depends upon ATP (50), yet an ATP-dependent, *in vitro* protein-degrading system was developed from rabbit reticulocytes only four years ago (14). Although fractionation of reticulocyte lysates holds considerable promise (9, 22), traditional biochemical approaches have proved difficult, and the mechanism underlying selective protein degradation remains obscure.

Development of large-scale microinjection procedures offers an alternate method to study intracellular protein degradation (17, 29, 47). Proteins can be modified and then introduced into cells to discover those features of protein structure significant in determining degradation rates. Moreover, injection of a specific, radio-iodinated protein allows one to determine the half-life, location, and size of the injected protein without

confusion from other labeled proteins. Using this approach, we have shown that specific, radio-iodinated proteins are degraded at different rates after injection into cultured mammalian cells (57) and that the selective degradation of bovine serum albumin (BSA) and pyruvate kinase occurs largely in the cytosol rather than within lysosomes (4). In similar studies it has been reported that the degradation of proteins microinjected into IMR-90 human diploid fibroblasts conforms to isoelectric and size correlations determined for endogenous proteins (32) and that injected RNase A is also degraded mainly in the cytosol rather than the lysosomes of IMR-90 fibroblasts (13). Injection procedures have also shown that hemoglobin is degraded after introduction into cultured Baby Hamster kidney (BHK) cells and that its degradation rate increases upon phenylhydrazine denaturation (21).

Here we examine the degradation of radio-iodinated im-

munoglobulin G molecules (IgGs) within HeLa cells. It is readily admitted that immunoglobulins are not typical intracellular proteins and that iodination may change their intracellular degradation rates (32, 35). Nevertheless, all proteins examined in these studies are IgGs or fragments thereof, and they are labeled in the same manner. Thus, we feel that they may legitimately be used to probe the specificity properties of intracellular protein degradation. Indeed, IgG molecules offer several distinct advantages for such studies. First, they have attractive structural features including variable isoelectric points with a constant conformation, two easily separable regions (Fab and Fc fragments), attached carbohydrate chains, and the existence of a variety of myeloma proteins which will prove useful in correlating subtle aspects of protein structure and degradation rates. Second, it has been shown that injection of antibody to diphtheria toxin or ricin protects cells upon subsequent challenge with these toxins (34, 54). This raises the possibility that injection of specific antibodies can be used to select for mutations within the degradative system. Finally, injection of antibodies to inhibit intracellular processes is becoming an increasingly popular experimental approach (2, 43). Information on the location and stability of IgG molecules within injected cells is valuable for designing and interpreting such experiments. Surprisingly, present estimates for the half-life of injected IgG molecules vary almost 20-fold (52, 56). For these reasons, we felt that examination of the stabilities and locations of IgG molecules and IgG fragments was warranted.

## MATERIALS AND METHODS

### *Isolation and Digestion of Rabbit Immunoglobulin G:*

Rabbit immunoglobulin G (IgG) was purified from four different pools of rabbit serum by ammonium sulfate precipitation and DEAE chromatography as described previously for goat IgG (39). Rabbit IgG was digested with papain, and the fragments were purified by gradient elution from carboxymethyl cellulose (Whatman CM52) as described by Porter (38). Rabbit IgG molecules were degraded with pepsin, as described by Nisonoff et al. (33), and purified by chromatography on Sephadex G-100. The identity of the purified fragments was confirmed by SDS acrylamide gel analysis and isoelectric focusing. In addition, >85% of  $^{125}\text{I}$ -labeled whole IgG and 70% of  $^{125}\text{I}$ -Fc fragments were bound by *Staphylococcus aureus* cells whereas <3% of  $^{125}\text{I}$ -Fab fragments were bound.

For one experiment rabbit IgG molecules were first radio-iodinated and then digested with papain. The labeled fragments were purified as described above, except that stepwise rather than gradient elution was employed and the purified fragments were concentrated by dialysis against Aquacide before loading into erythrocytes. These modifications in the purification procedure produced no apparent difference in fragment stability (see below Table I).

**Myeloma Proteins:** The human myeloma protein, Mcg, and four mouse monoclonal antibodies against fluorescein were kindly provided by K. Ely and A. Edmundson (University of Utah).

**Iodination of Proteins:** Proteins were iodinated by the lactoperoxidase method as previously described (41) or by chloramine-T as before (39). Typical specific activities ranged from  $5 \times 10^5$  to  $5 \times 10^6$  cpm/ $\mu\text{g}$  corresponding to substitution ratios of 0.1 to 1.0 mol of  $^{125}\text{I}$ /mol of protein. All iodinated proteins were used within 1 d of iodination.

**Cell Culture:** The heteroploid human line, D98/AH2, was obtained from the American Type Culture Collection and grown in Ham's F12 medium supplemented to 5% with a 3:1 mixture of calf serum and fetal calf serum. Cells were collected for fusion and routinely transferred by removal from plastic culture flasks with 0.1% trypsin in  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ -free saline. Cell number was determined by hemacytometry.

**Microinjection:** Methods for introducing proteins into erythrocytes and the general microinjection procedure have been thoroughly described (48). In our experiments ~15% of the added IgG and 30% of the added fragments were retained by erythrocytes. Fusions were performed in 0.5 ml of Tris-saline, pH 7.4, containing 1 mM  $\text{Mn}^{++}$ ,  $5-15 \times 10^6$  D98/AH2 cells,  $5-15 \times 10^6$  loaded erythrocytes and 600 to 1,000 HAU of UV-inactivated Sendai virus. Between 0.4% and 1.2% of the loaded protein was transferred from erythrocytes to HeLa cells. This corresponds to injection of 25-75% of the HeLa cells (40). An injected HeLa cell, which increases in volume ~6% and in plasma membrane area by 20%, received between  $10^4$  and  $8 \times 10^4$   $^{125}\text{I}$ -labeled IgG molecules or IgG fragments in the studies reported here.

**Measurement of Rates of Degradation:** Almost all methods for measuring intracellular protein degradation in mammalian cells in culture involve determining the rate of release of labeled amino acids from prelabeled or microinjected cells. This rate is then compared to total isotope incorporated into cellular protein or microinjected into the cells to yield rates of protein degradation generally expressed as percent protein degraded per unit time. The fact that proteins are also released into the medium by processes such as secretion or cell death complicates the above analysis. Some investigators have attempted to correct for protein release (24, 49), whereas others have ignored it (32, 52, 55, 56, 57).

In the studies presented below, we found that substantial amounts of  $^{125}\text{I}$  were released as TCA-insoluble material from monolayers of HeLa cell injected with  $^{125}\text{I}$ -IgG or  $^{125}\text{I}$ -IgG fragments. To determine whether the released TCA-insoluble material was intact protein, we removed mitotic cells from the medium by centrifugation at 1,000 g for 10 min, and samples of culture medium were then dialyzed against 10 mM Tris, pH 7.4, containing 10 mM NaCl, lyophilized, reconstituted to one-tenth the volume with distilled water, and chromatographed on a Sephadex G150 column in 100 mM Tris, pH 7.4, containing 100 mM NaCl. Virtually all TCA-insoluble  $^{125}\text{I}$  in the medium chromatographed as the original IgG or IgG fragment depending upon the experiment. No degradation of the released protein was observed when culture medium was removed from injected cells and incubated at 37°C in empty culture flasks or over monolayers of uninjected HeLa cells.

Culture medium overlying injected HeLa cells was also analyzed by chromatography on Whatman 3 MM paper (Whatman Inc., Paper Div., Clifton, NJ) as previously described (57). Radio-iodine was present at the origin and at positions corresponding to iodotyrosine and  $\text{I}^-$ . The fraction of  $^{125}\text{I}$  at the origin corresponded to the TCA-insoluble  $^{125}\text{I}$  present, and almost all remaining  $^{125}\text{I}$  was present as iodotyrosine; <10% of the TCA-soluble  $^{125}\text{I}$  was  $\text{I}^-$  (data not shown).

Thus after radio-iodinated IgG and IgG fragments are injected into HeLa cells,  $^{125}\text{I}$  is released to the culture medium as  $^{125}\text{I}$ -iodotyrosine upon degradation of the injected protein and as  $^{125}\text{I}$ -labeled protein by processes not yet understood. Direct microscopic examination of the culture medium revealed that cells unable to exclude trypan blue, i.e., dead cells, could account for at least half and perhaps all of the released protein.<sup>1</sup> Because labeled protein is removed from the pool of intracellular protein available for degradation upon release to the medium, comparing the rate of iodotyrosine production to the total TCA-insoluble protein present is clearly invalid. On the other hand, one cannot necessarily exclude the intact protein in the medium since it may have been part of the intracellular pool for some portion of the time over which iodotyrosine production was measured.

Analysis of numerous experiments revealed that, except for the first few hours after injection,<sup>2</sup> there was a constant relation between  $^{125}\text{I}$ -iodotyrosine and  $^{125}\text{I}$ -protein released from HeLa monolayers after injection with  $^{125}\text{I}$ -IgG or  $^{125}\text{I}$ -labeled IgG fragments (see Fig. 1).<sup>3</sup> This relationship greatly simplifies the

<sup>1</sup> This has been estimated as follows. We can find 8-10% dead cells in the medium at 24 h after injection. Assuming death to be first-order and assuming that only injected cells die, one can calculate a death rate of 0.4%/h. Rates of intact protein release vary from 0.6%/h for human IgG to 0.8%/h for rabbit Fc fragment. If there were preferential death of those HeLa cells which fuse with multiple erythrocytes, one might account for all isotope loss by cell death. On the other hand, if both injected and noninjected cells die in our system, then cell death alone cannot account for release of intact protein to the medium.

<sup>2</sup> Analysis is complicated during the first several hours after injection, for two reasons. It has been shown that rates of protein degradation are elevated by as much as 40% after fusion, but that they return to normal within 2-4 h (21, 32). A more significant complication results from the fact that our cultures are still contaminated by loaded erythrocytes during the period of cell attachment, and release of  $^{125}\text{I}$ -proteins from lysing erythrocytes results in vast overestimation of the rate of release of intact protein from injected HeLa cells.

<sup>3</sup> This surprisingly constant relationship between degradation and release of intact protein suggests that processes other than cell death may underlie the release of intact protein from injected HeLa cells. For example, cells can transfer cytosolic proteins to lysosomes by autophagy. If this transfer goes by way of the recently discovered "acid compartment," then cytosolic proteins might enter a vesicle shuttle between this compartment and the cell surface (see reference 30 for a recent discussion on vesicle traffic). Another interesting possibility is that cells simply exfoliate abnormal proteins in vesicles (26, 27, 51). One can imagine that abnormal proteins interact with actin or other cytoskeletal proteins and induce exfoliative release. The entry of intact iodinated proteins into the medium could, therefore, reflect cell death, diversion of the contents of autophagic vacuoles, or exfoliation, and we are presently attempting to quantitate these processes.

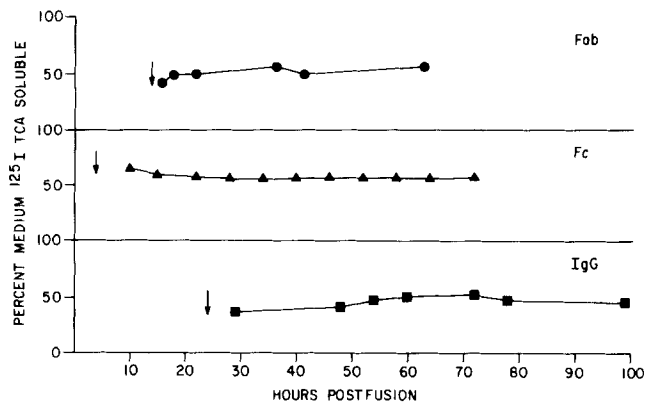


FIGURE 1 Ratio of TCA-soluble to TCA-precipitable  $^{125}\text{I}$  in the culture medium overlaying HeLa cells injected with radio-iodinated IgG and IgG fragments. HeLa cells were injected with  $^{125}\text{I}$ -IgG,  $^{125}\text{I}$ -Fab, or  $^{125}\text{I}$ -Fc fragments, allowed to plate onto culture flasks, and subsequently rinsed with warm medium (vertical arrow). At the indicated times thereafter, the ratio of TCA-soluble to total  $^{125}\text{I}$  present in the culture medium was determined as described under Materials and Methods. The relatively constant ratios facilitate calculations of the degradation rates for injected proteins.

calculation of the rates of degradation of the injected proteins because it permits one to express the first-order rate constant for the loss of  $^{125}\text{I}$  from HeLa monolayers ( $K_{\text{loss}} = K_i$ ) as the sum of the two rate constants,  $K_d$  (degradation) and  $K_r$  (release). Further, it follows that if the deposition of  $^{125}\text{I}$  into the culture medium is a first-order process and that the ratio of TCA-soluble  $^{125}\text{I}$  ([S]) to TCA-precipitable  $^{125}\text{I}$  ([P]) is a constant, then the ratio  $K_d:K_r$  is also a constant and directly proportional to the ratio [S]:[P]. That is: (1)  $K_i = K_d + K_r$ , and (2)  $\frac{K_d}{K_r} = \frac{[S]}{[P]}$ .

Using the first-order rate equation  $A = A_0 e^{-Kt}$ ,  $K_d$  can be expressed in terms of [S] and [P]:

$$K_d = \frac{\ln A_0/A}{t(1 + [P]/[S])}$$

provided  $K_i$  is first order, the ratio [P]/[S] is constant, and the  $^{125}\text{I}$  cpm in the medium at  $t = 0$  is zero.  $A_0$  is the  $^{125}\text{I}$  in the HeLa monolayer at the beginning of the experiment and  $A$  is the  $^{125}\text{I}$  in the HeLa monolayer at any time,  $t$ .

Using this equation, we calculated values of  $K_d$  for each sample in an experiment, and an average  $K_d$  was then determined. Since half-lives ( $t_{1/2}$ ) are more easily comprehended than  $K_d$  values, text and tabular entries are expressed as  $t_{1/2}$  in hours obtained from the equation,  $t_{1/2} = 0.693/K_d$ . Graphs contain direct measures of  $K_{\text{loss}}$  normalized by expression as percent  $^{125}\text{I}$  remaining in the monolayer as a function of time.

Primary data were collected as follows. After fusion with erythrocytes loaded with  $^{125}\text{I}$ -labeled protein, HeLa cells were plated into numerous 25- or 75-cm<sup>2</sup> T-flasks and allowed to attach for 6 to 18 h. Cell monolayers were then rinsed thoroughly with prewarmed medium and incubated in 2-8 ml of medium for the duration of the measurement period. At selected times the medium was removed and combined with a 1 ml of cold PBS rinse, and the cell monolayer was dissolved in 1 ml of 1 N NaOH. Total  $^{125}\text{I}$  present in the medium and NaOH fraction was determined on a Beckman Autogamma 4000 (Beckman Instruments, Inc., Palo Alto, CA), and 100% TCA was then added to two 1-ml aliquots of the mixture of PBS and medium to produce a final TCA concentration of 10%. After 15 minutes on ice, the TCA-treated medium was centrifuged in an Eppendorf centrifuge (Model 5412) (Brinkmann Instruments, Inc., Westbury, NY), and the  $^{125}\text{I}$  present in the supernatant (TCA soluble  $^{125}\text{I}$ ) was determined. The partition of  $^{125}\text{I}$  between medium and monolayer permitted direct measurement of  $K_{\text{loss}}$  and the ratio of TCA-soluble to TCA-insoluble  $^{125}\text{I}$  in the medium permitted calculation of  $K_d$  as described above. Greater than 97% of the  $^{125}\text{I}$  in the NaOH extract was TCA-insoluble. Within typical experiments, total  $^{125}\text{I}$  recovered from each flask varied <5%.

**SDS Acrylamide Gel Electrophoresis:** Proteins were analyzed on SDS polyacrylamide gels as described by Laemmli (28). Samples were electrophoresed in 15% gels for 600-800 V-h. After electrophoresis the gels were dried onto Whatman 3 MM paper without prior fixation. After exposure to x-ray film the dried gels were cut into 0.4-cm segments, and the distribution of  $^{125}\text{I}$  was determined on a Beckman Autogamma 4000.

**Isoelectric Focusing:** Proteins were extracted by suspending frozen and thawed cell pellets three times in 50  $\mu\text{l}$  of 10 mM Tris, pH 7.4, 10 mM MgCl<sub>2</sub>,

30 mM KCl, 1% Triton X-100, and 1 mM dithiothreitol. After each suspension the mixture was clarified by centrifugation for 5 min on an Eppendorf centrifuge. The three supernatants were combined and analyzed on isoelectric focusing gels by slight modification of the procedure of Righetti and Chillemi (42). For whole IgG and Fab fragments, >70% of the injected proteins was recovered in the extracts; for Fc fragments, recovery was only 50%.

Vertical slab gels composed of 4.5% acrylamide, 0.12% bis-acrylamide, 12.5% sucrose, and 2% LKB ampholines, pH 5-8 or pH 3-10, were electrophoresed between cathode and anode buffers consisting of 0.02 M NaOH and 0.2% H<sub>2</sub>SO<sub>4</sub>, respectively. Gels were run at 4°C for 4 h at 200 V. After electrophoresis, gels were dried onto 3 MM paper, gel lanes were cut into 5-mm segments, and the distribution of  $^{125}\text{I}$  was determined in a Beckman Autogamma 4000.

**Autoradiography:** Autoradiography of whole cells and thin sections was performed as previously described (41).

**Materials:**  $^{125}\text{I}$  was obtained from Amersham Corp. (Arlington Heights, IL). Ampholines were purchased from LKB Instruments (Gaithersburg, MD). All tissue culture supplies were obtained from Flow Laboratories Inc. (Rockville, MD). Papain, pepsin and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

## RESULTS

### Heavy and Light Chains of IgG Are Degraded at the Same Rate

It has been suggested that dissociation into subunits could be an initial event in protein degradation (46). Because IgG molecules are composed of different-size heavy and light chains, whether this suggestion is correct might be determined by measuring the relative concentration of each chain within injected cells.  $^{125}\text{I}$ -labeled IgG molecules were injected into HeLa cells and, at various times thereafter, intracellular radioactivity was analyzed on SDS-acrylamide gels (see (Fig. 2). There was a progressive decrease in intracellular  $^{125}\text{I}$  within HeLa cells, but there was no change in the relative amount of  $^{125}\text{I}$  in heavy and light chains (Fig. 3). If heavy and light chains dissociate before degradation within HeLa cells, then the chains are degraded at the same rate. Assuming that dissociation does not occur before IgG degradation, the constant ratio of heavy and light chains indicates that, once proteolysis is initiated, the entire molecule is degraded.

### Isoelectric Points of IgGs and Their Degradation Rates

During the past ten years there have been numerous attempts to correlate protein structure with intracellular degradation rates. Evidence has been presented indicating that larger proteins with larger subunits are degraded faster than those with smaller subunits (11, 12, 20) and that negatively charged pro-

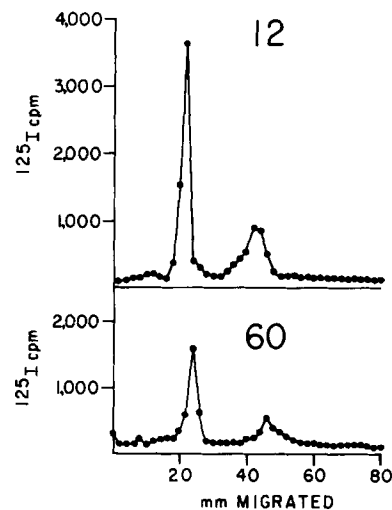


FIGURE 2 SDS acrylamide electrophoresis of injected  $^{125}\text{I}$ -IgG molecules. HeLa cells were injected with  $^{125}\text{I}$ -IgG, and intracellular proteins were analyzed on SDS acrylamide gels. The electrophoretic patterns show that the remaining intracellular  $^{125}\text{I}$ -IgG molecules were composed of intact heavy and light chains at 12 and 60 h after injections.

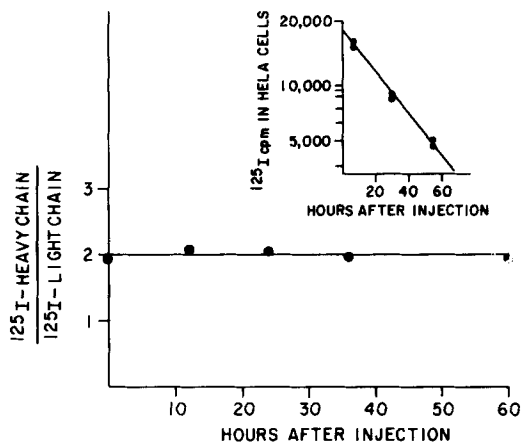


FIGURE 3 Chain composition of injected  $^{125}\text{I}$ -IgG. Radio-iodinated IgG was injected into HeLa cells and, at various times thereafter, the remaining intracellular  $^{125}\text{I}$ -IgG molecules were analyzed on SDS acrylamide gels. Ratios of heavy to light chains, computed from electrophoretic patterns similar to those in Fig. 2, are presented above. The inset shows the rate of loss of  $^{125}\text{I}$  from injected HeLa cell in the experiment used to determine the ratio of heavy to light chain.

teins are degraded faster than positively charged proteins (1, 12). In contrast, several other studies reveal no significant correlation between degradation rate and protein charge or subunit molecular weight (6, 31, 44). Thus, it is not clear that the proposed correlations between structure and degradation are correct for all cellular proteins or cell types.

IgG molecules offer a unique opportunity to study the relationship between the isoelectric point of a protein and its degradation rate. Although sequence diversity in the variable regions of heavy and light chains produces molecules with different isoelectric points, the proteins still have equivalent overall conformations (37). Such a spectrum of molecules permits one to test whether degradation rates are directly related to surface charge or whether some other characteristic of protein structure, perhaps itself related to charge, is important.

HeLa cells were injected with  $^{125}\text{I}$ -IgG, collected at various times thereafter, and the remaining intracellular IgG molecules were analyzed on pH five to eight isoelectric focusing gels. For comparison, cells injected with  $^{125}\text{I}$ -BSA were analyzed in parallel. As shown in Fig. 4 the initial isoelectric profile of  $^{125}\text{I}$ -IgG was much broader than that of  $^{125}\text{I}$ -BSA. With time there was an increased proportion of negatively charged IgG molecules, whereas no change was observed in the isoelectric profile of injected  $^{125}\text{I}$ -BSA. The shift in the isoelectric pattern for whole  $^{125}\text{I}$ -IgG molecules, observed in three separate experiments, indicates either that positively-charged IgGs are degraded more rapidly, or that the isoelectric points of positively charged rabbit IgGs are changed by protein modifications.

To test the latter possibility, we measured the isoelectric profiles of two mouse monoclonal antibodies after injection into HeLa cells. According to the data in Fig. 5 there was no change in the isoelectric point of monoclonal MC 19-1. Results with monoclonal MC 4-4 are equivocal since ~30% of the recovered  $^{125}\text{I}$  focused as a smear at lower pHs. These results admit the possibility that the isoelectric point of some molecules of monoclonal MC 4-4 were shifted by modification, although with this explanation it is difficult to understand why all injected molecules were not charge-shifted or why modification was not progressive with time.

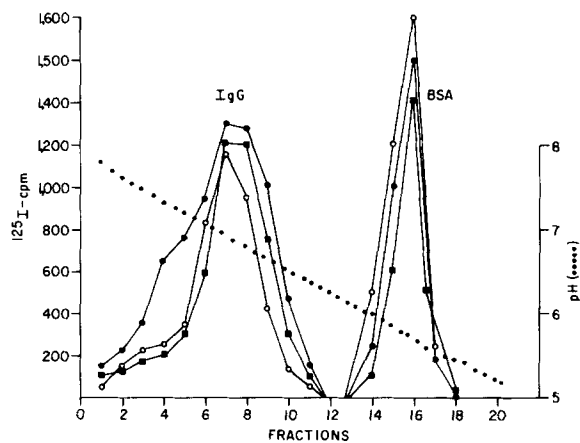


FIGURE 4 Isoelectric profiles of  $^{125}\text{I}$ -IgG and  $^{125}\text{I}$ -BSA after injection into HeLa cells. HeLa cells were injected with  $^{125}\text{I}$ -IgG or  $^{125}\text{I}$ -BSA and, at various times after injection, the intracellular proteins were extracted and analyzed on pH 5-8 isoelectric focusing gels. For  $^{125}\text{I}$ -IgG, which focused between pH 7.5 and 6.0, solid circles denote 3 h after injection, open circles denote 31 h after injection, and solid squares denote 67 h after injection. The isoelectric profile of uninjected  $^{125}\text{I}$ -IgG was virtually indistinguishable from the pattern observed at 3 h. It has been omitted to prevent excessive crowding of the figure. For  $^{125}\text{I}$ -BSA, which focused at pH 5.6, open circles denote 0.5 h after injection, closed circles denote 21 h after injection, and solid squares denote 54 h after injection.

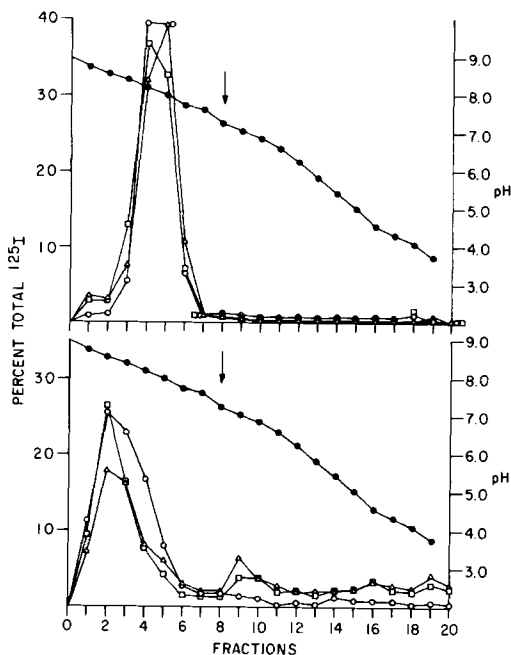


FIGURE 5 Isoelectric profiles for two mouse monoclonal antibodies after injection into HeLa cells. HeLa cells were injected with  $^{125}\text{I}$ -MC 19-1 (upper panel) or  $^{125}\text{I}$ -MC 4-4 (lower panel), and intracellular proteins were analyzed on pH 3-10 isoelectric focusing gels at 0 h (O), 18 h ( $\Delta$ ) or 40 h ( $\square$ ) for MC 19-1 and at 0 h (O), 18 h ( $\Delta$ ) and 23 h ( $\square$ ) for MC 4-4. Vertical arrows denoted the position of hemoglobin after isoelectric focusing.

### Mouse Monoclonal Antibodies Are Degraded at Different Rates

It was observed during the studies on isoelectric focusing of mouse monoclonals that MC 4-4 was degraded eightfold faster than MC 19-1. Since MC 19-1 contains a heavy chain of  $\gamma_1$  class and MC 4-4 contains a heavy chain of  $\gamma_2$  class, we

examined the intracellular stability of an additional monoclonal with each class of heavy chain. As shown in Table I, both monoclonals with  $\gamma_1$  heavy chains were much more stable than those with  $\gamma_2$  heavy chains. It is not clear that this differential stability will extend to all mouse monoclonal antibodies with  $\gamma_1$  and  $\gamma_2$  heavy chains, but those who might inject monoclonal antibodies to inhibit intracellular processes should be aware of this possibility.

### Degradation of Intact IgG and IgG Fragments

The ability of papain and pepsin to cleave IgG molecules into well characterized Fab, Fab<sub>2</sub>, and Fc fragments allowed us to ask whether any of these fragments contain key structural features which determine the rate of degradation of intact IgG molecules within HeLa cells. The various fragments of rabbit IgG were prepared, purified, radio-iodinated, and their rates of degradation were determined after injection into HeLa cells. According to the data in Fig. 6 and Table I, intact IgG molecules, Fab<sub>2</sub> and Fc fragments were degraded at similar rates, whereas Fab fragments were significantly more stable. Although these results indicate that neither Fc nor Fab regions contains a dominant feature responsible for the degradation of intact IgG molecules, they suggest that the Fc fragment may elicit degradation more frequently. The comparable stabilities of IgG fragments and the intact molecule provide good evidence that proteolytic cleavage at the hinge region of rabbit IgG is not the rate-limiting event for IgG degradation within HeLa cells. Further evidence that hinge regions play little role in the intracellular degradation of IgG was obtained by comparing the degradation of normal human IgG and Mcg, a human myeloma protein which lacks 15 amino acids in the hinge (16). The data in Table I show that the intracellular half-life of Mcg was virtually identical to that of normal bulk human IgG.

### Isoelectric Profiles and the Degradation Rates of IgG Fragments

The Fc portion of rabbit IgG molecules contains carbohydrate moieties which terminate either in the neutral sugar, *N*-acetylglucosamine, or in negatively charged sialic acid (53). The presence of alternate terminal sugars provides a possible explanation for the more rapid degradation of positively charged rabbit IgG molecules. That is, one can imagine that

TABLE I  
Intracellular Stability of IgGs and IgG Fragments

Species	Protein	Half-life <i>h</i>	Mean $\pm$ SD
Rabbit	IgG*	67, 82, 69, 114	83 $\pm$ 22
Rabbit	Fab	90, 112 $\ddagger$ , 106, 111	105 $\pm$ 10
Rabbit	Fc	47, 36, 78, 61, 66 $\ddagger$ , 75, 64	61 $\pm$ 15
Rabbit	Fab <sub>2</sub>	78	
Human	IgG*	91	
Human	Myeloma Mcg	93	
Mouse	Myeloma 19-1 ( $\gamma_1$ )	92, 74 $\S$	
Mouse	Myeloma 20-3 ( $\gamma_1$ )	85	
Mouse	Myeloma 4-4 ( $\gamma_2$ )	9 $\S$ , 13	
Mouse	Myeloma 6-19-1 ( $\gamma_2$ )	11	

\* IgG refers to bulk IgG purified from serum.

$\ddagger$  These half-lives were obtained from fragments generated after iodination of the parent IgG molecule.

$\S$  These half-lives were obtained from proteins labeled by chloramine-T radioiodination.

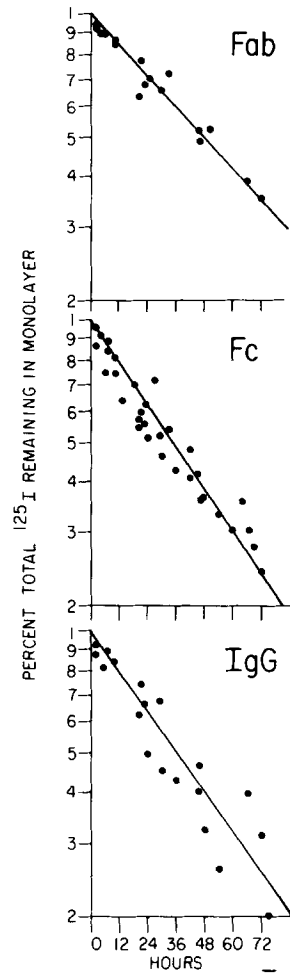


FIGURE 6 Loss of  $^{125}\text{I}$  from HeLa cells injected with  $^{125}\text{I}$ -IgG and  $^{125}\text{I}$ -labeled IgG fragments. Fab and Fc fragments of rabbit IgG were prepared and purified as described in Materials and Methods. The purified fragments and starting IgG were then radio-iodinated and injected into HeLa cells. Loss of  $^{125}\text{I}$  from injected cells was measured as described under Materials and Methods, and the data presented above are composites from three independent experiments for each protein.

sialic acid might shield a site normally recognized by the HeLa degradation system, or, alternatively, *N*-acetylglucosamine might itself be recognized by the degradative system. The availability of separated Fab fragments allowed us to re-examine the relation between degradation rate and isoelectric point without the possible complication of attached carbohydrate chains.  $^{125}\text{I}$ -labeled Fab fragments were injected into HeLa cells, the injected cells were collected at several times during the following 100 h, and the intracellular Fab fragments remaining in each sample of cells were analyzed on isoelectric focusing gels. It is evident from the profiles presented in Fig. 7 that neither negatively charged nor positively charged Fab fragments was preferentially degraded. Similar results were obtained with Fab<sub>2</sub> fragments (Fig. 8).

The situation was more complicated for Fc fragments where three prominent bands were observed after isoelectric focusing. Of these, the fragment with an isoelectric point of 6.9 appeared to be rapidly degraded in contrast to fragments with isoelectric points of 7.3 and 6.5 (Fig. 9). However, like the results from intact IgG injection, the possibility that protein modification or unequal extraction accounts for the altered patterns cannot be entirely excluded.

### Distribution of Intact IgG, Fab, and Fc Fragments within HeLa Cells

Whether injected antibodies can gain access to the various cellular compartments is of considerable interest to those investigators who might attempt to inhibit cellular processes by

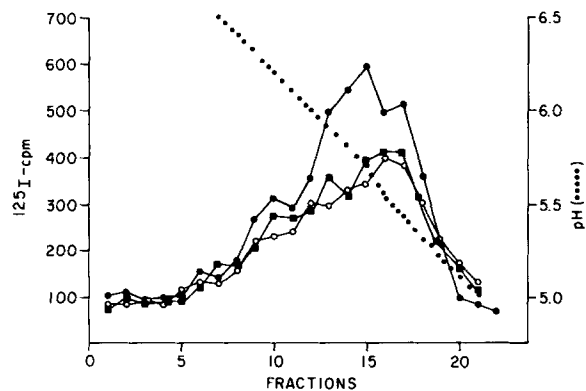


FIGURE 7 Isoelectric profiles of  $^{125}\text{I}$ -Fab fragments after injection into HeLa cells. HeLa cells were injected with  $^{125}\text{I}$ -Fab fragments, and intracellular proteins were analyzed on pH 5-8 isoelectric focusing gels at 5 (●), 55 (■), or 100 h (○) after injection.

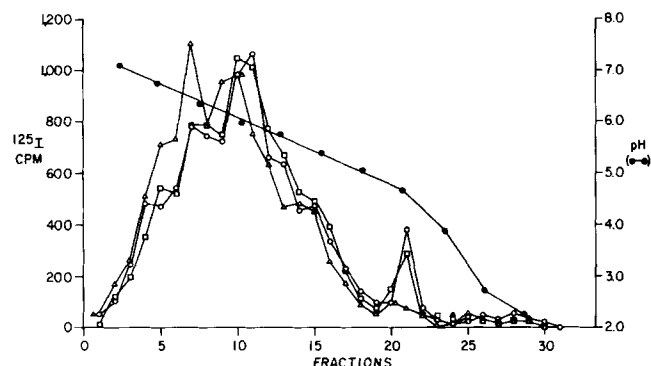


FIGURE 8 Isoelectric profiles of  $^{125}\text{I}$ -Fab<sub>2</sub> fragments after injection into HeLa cells. HeLa cells were injected with  $^{125}\text{I}$ -Fab<sub>2</sub> fragments, the intracellular proteins were analyzed on pH 5-8 isoelectric focusing gels after extraction at 5 (○), 20 (□), or 70 h (Δ) following fusion. The profile of uninjected  $^{125}\text{I}$ -Fab<sub>2</sub> fragments was not significantly different from the patterns shown above; it has been omitted to reduce crowding.

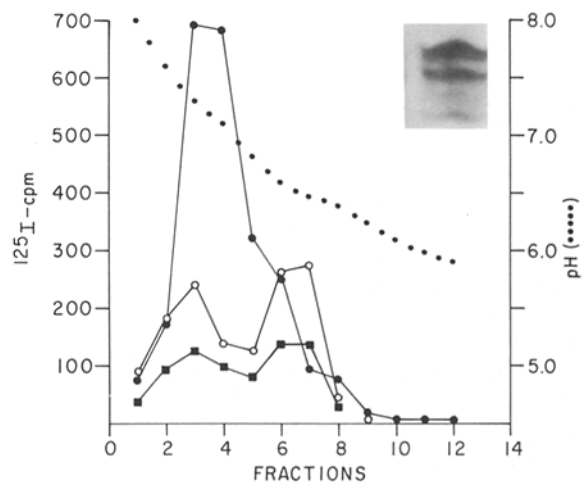


FIGURE 9 Isoelectric profiles of  $^{125}\text{I}$ -Fc fragments after injection into HeLa cells. HeLa cells were injected with  $^{125}\text{I}$ -Fc fragments, and intracellular proteins were analyzed on pH 5-8 focusing gels after extraction at 0.5 h (●), 45 (○) or 91 h (■) after injection. The isoelectric profile observed for uninjected  $^{125}\text{I}$ -Fc fragments is virtually identical to that observed at 0.5 h. The inset in the upper right corner shows an autoradiogram of  $^{125}\text{I}$ -Fc. Individual bands are clearly visible.

antibody injection. Because a number of antibody injection studies have focused on nuclear proteins, we have examined the nuclear-cytoplasmic partitioning of IgG and IgG fragments after erythrocyte-mediated injection into HeLa cells.

Autoradiographic studies on HeLa cells plated onto cover slips and fixed 24 h after injection with  $^{125}\text{I}$ -IgG or  $^{125}\text{I}$ -labeled IgG fragments revealed a difference in the intracellular distribution of the injected proteins. The ratio of nuclear grain density to cytoplasmic grain density was 1.35 for cells injected with Fab or Fc fragments, whereas this ratio was 0.95 for cells injected with intact IgG. These results suggested either that intact IgGs were partially excluded from HeLa nuclei or that Fab and Fc fragments partially concentrated there. To distinguish between these two possibilities, we performed further autoradiographic analysis on thin sections of injected cells (Fig. 10). These data are summarized in Table II, and they strongly suggest that IgG molecules are excluded from HeLa nuclei.

## DISCUSSION

Half-lives reported for IgG molecules injected into cultured mammalian cells vary from 7.5 h in Ehrlich ascites tumor cells (56) to 160 h in murine erythroleukemia cells (52). This substantial range could reflect differences in the protein degradation pathways among the various cell lines used. Alternatively, these large differences might reflect methodological problems particularly since in several studies, including one from this laboratory (57), degradation was incorrectly measured simply as loss of  $^{125}\text{I}$  from injected cells. The present study has shown that a considerable portion of microinjected  $^{125}\text{I}$ -IgG can be lost as intact protein, so previous values are almost surely in error. We have corrected for loss of intact protein, and we therefore consider the 70-90 h half-lives reported here for  $\gamma_1$  mouse IgG, bulk rabbit IgG, and bulk human IgG to be more accurate estimates of the intracellular stability of IgG molecules. These should be considered minimal estimates of stability since radioiodination may increase the degradation rate of proteins (32, 35). The stability of IgG observed in HeLa cells will probably be obtained in most cultured cell lines since we previously found that the BSA degradation varied less than twofold among eight different cell lines (57), and we have found that the degradation of a  $\gamma_1$  mouse monoclonal antibody varies <20% among five cell lines from three species (unpublished observations).

We have shown before that  $^{14}\text{C}$ -sucrose-labeled BSA and pyruvate kinase are degraded largely in the cytosol after injection into L929 cells (4). Similar attempts to determine the cellular compartment in which IgG molecules are degraded proved unsuccessful because massive precipitation occurred

TABLE II  
Distribution of Injected  $^{125}\text{I}$ -IgG and  $^{125}\text{I}$ -labeled IgG Fragments within HeLa Cells\*

Protein	Hours after fusion	Cells examined	Grains per 33 $\mu\text{m}^2$		
			Cytoplasm	Nucleus	Nucleus/cytoplasm
Fab fragment	0	48	6.0	7.4	1.2
Fc fragment	0	50	2.9	2.9	1.0
Intact IgG	0	41	11.7	3.8	0.3
Intact IgG	17	26	7.7	2.3	0.3

\* HeLa cells were injected with  $^{125}\text{I}$ -IgG,  $^{125}\text{I}$ -Fab, or  $^{125}\text{I}$ -Fc fragments and fixed immediately after fusion or 17 h later. The injected cells were embedded in Epon; 1-2- $\mu\text{m}$  sections were cut and overlaid with autoradiographic emulsion. Autoradiograms were developed after 5 or 9 d, and grain densities over nuclei and cytoplasm were determined under oil immersion with a 40  $\times$  40 eyepiece reticle with unit size 5.75  $\times$  5.75  $\mu\text{m}$ .

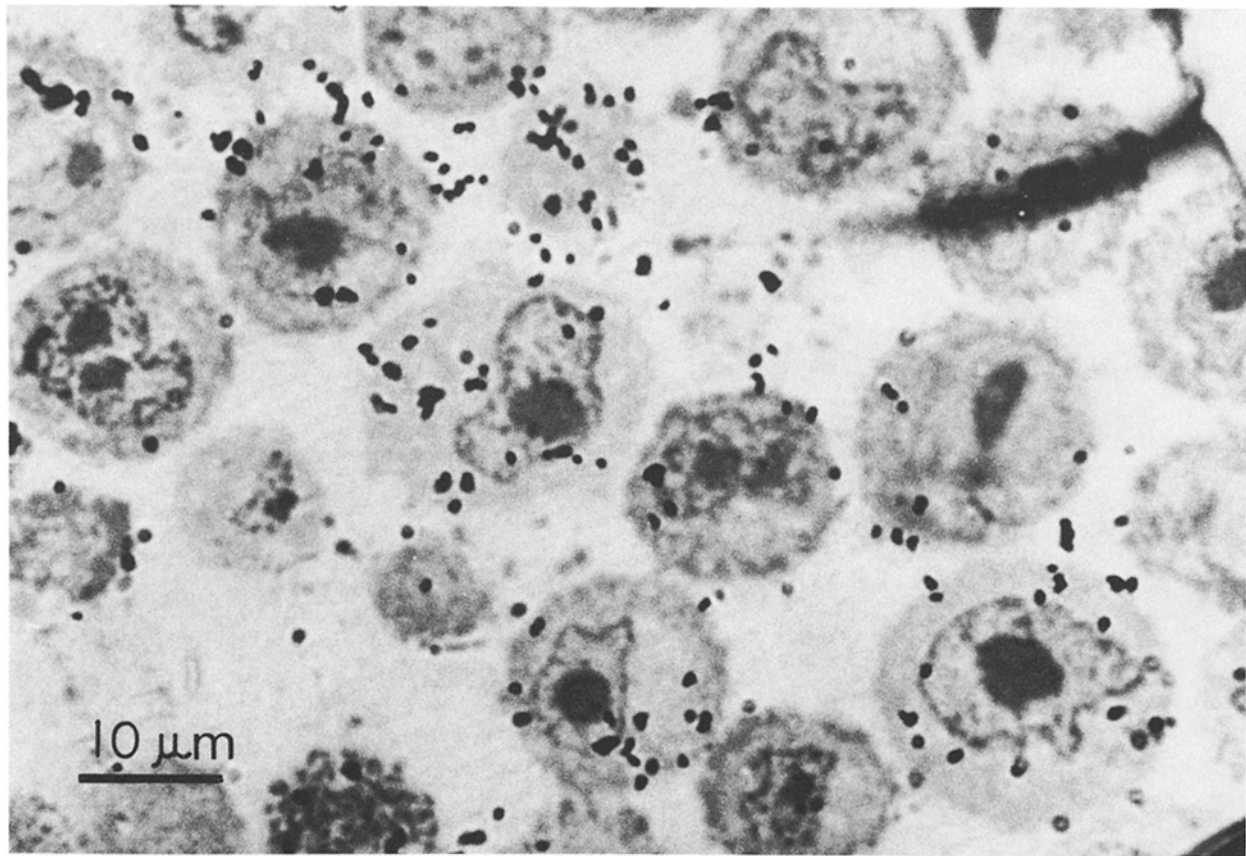


FIGURE 10 Autoradiogram of sectioned HeLa cells injected with intact  $^{125}\text{I}$ -IgG. The cells shown were injected with intact  $^{125}\text{I}$ -IgG 17 h before fixation. Note that almost all grains are over cytoplasm rather than nuclei. Autoradiographic exposure was 9 d.

during the sucrose coupling reaction. However, we have found that Arrhenius plots for the degradation of injected  $^{125}\text{I}$ -Fc or  $^{125}\text{I}$ -BSA show no transitions from 7° to 37°C, and the activation energy for the degradation of both proteins is 25 Kcal (R. Hough and M. Rechsteiner, manuscript in preparation). We have also found that the degradation of both  $^{125}\text{I}$ -pyruvate kinase and  $^{125}\text{I}$ -IgG is inhibited only 25–35% by the lysosomotropic agents, ammonia, and chloroquine (K. V. Rote and M. Rechsteiner, manuscript submitted for publication; and R. Hough unpublished observation). These similarities between the degradation of IgG, Fc fragments, BSA, and pyruvate kinase suggest that all of these proteins are degraded largely in the cytosol. Moreover, it has recently been shown that the Fc fragment of rabbit IgG is preferentially degraded in rat fibroblast lysosomes (49), but we did not observe preferential stability of IgG light chains (Fig. 2) which further supports nonlysosomal degradation of injected IgG molecules.

A major goal of the present study has been to identify structural features of IgG molecules which affect their rates of intracellular degradation. A relevant question in this regard is whether proteolysis is elicited by features on the Fab or Fc portions of the IgG molecule. The data in Table I show that Fc, Fab<sub>2</sub>, and intact IgG molecules were degraded with similar 60–80-h half-lives whereas the 110-h half-life of Fab fragments was significantly longer. The similar half-lives of Fab<sub>2</sub> and Fc fragments are not consistent with their being a major determinant for IgG degradation in either of these two regions of the intact molecule. Nevertheless, the increased stability of Fab fragments does suggest that the degradation of intact IgGs within HeLa cells is initiated more often by a feature(s) on the Fc fragment.

Isoelectric point is a second aspect of IgG structure which

we have examined with respect to degradation rate. Following injection, positively charged IgG molecules disappeared faster than their negative counterparts (Fig. 4), and a specific subset of Fc fragments with isoelectric points around pH 6.9 disappeared faster than Fc fragments with other pIs (Fig. 9). Although we cannot exclude the possibility that modification of the injected proteins is the basis for the altered isoelectric profiles, it is very likely that the changing patterns reflect differential degradation. Fab and Fab<sub>2</sub> fragments, which presumably possess very similar overall conformations and a spectrum of isoelectric points, offered a unique opportunity to correlate surface charge and degradation rate. Yet, in two experiments with each fragment there was no shift in isoelectric profile. Thus, our results with injected IgG molecules and fragments indicate that aspects other than surface charge are more important in determining the degradation rates of these proteins within cells.

The final aspect of these studies deals with the intracellular distribution of injected IgG molecules and fragments. While this is not directly related to protein degradation, it is of some interest to those investigators who might inject antibodies to inhibit intracellular functions. There is abundant evidence that certain features of a protein's structure (for example, leader or transit peptides, phosphomannose residues) can determine in which intracellular compartment the protein will accumulate (5, 8, 23, 25, 45). Whether there are specific signals which direct proteins to the nucleus remains unresolved. DeRobertis et al. (10) injected a mixture of  $^{125}\text{I}$ -labeled nonhistone chromosomal proteins into *Xenopus* oocytes and found a striking accumulation of the injected proteins within the germinal vesicle. They suggest that nuclear proteins might possess a common feature responsible for entry into the nucleus. On the other hand, there



are studies which suggest that size primarily determines whether proteins enter the nucleus (15, 36). Results from the present study support the latter hypothesis. Whereas Fab and Fc fragments distributed evenly between nucleus and cytoplasm, intact IgG was excluded from the nucleus (Table II). This exclusion was probably complete since the apparent distribution did not change during a 17-h period following fusion. Moreover, two other studies suggest that intact IgG molecules do not readily cross the nuclear membrane. Capecchi (7) found that  $^{125}\text{I}$ -IgG molecules remained within L929 nuclei after microneedle injection into that compartment, and D. Stacey (personal communication) observed that rhodamine-labeled IgGs remained in the cytoplasm of HeLa cells for at least 8 h after direct microneedle injection. It is likely, then, that the grains observed over HeLa nuclei in this study represent scatter from labeled IgG molecules present in the cytoplasm. If intact IgGs are indeed excluded from HeLa nuclei, this would indicate that protein size is the major barrier to entry into the nucleus since it is quite unlikely that an appropriate entry signal was generated on both Fab and Fc fragments by papain cleavage. Moreover, if intact IgG molecules are excluded from nuclei because of some structural feature of either the Fab or Fc fragment, then that fragment should have remained in the cytoplasm as well.

In summary, while the mechanism underlying the degradation of injected IgG molecules remains obscure, several features of the process have emerged from the present studies. Proteolysis in the hinge region of injected IgG molecules is clearly not the rate-limiting event in their eventual degradation within HeLa cells. If it were, we should have seen rapid degradation of Fab and Fc fragments. Second, the observation that Fc fragments and whole IgG molecules are degraded at similar rates and faster than Fab fragments suggests that Fc fragments contain features more often recognized by the HeLa degradative system. Third, the fact that heavy and light chains are degraded at the same rate rules out an intracellular protease that acts like pepsin and degrades only the Fc fragment. Fourth, isoelectric point is relatively unimportant in determining the intracellular degradation rates of intact IgG molecules, Fab, Fab<sub>2</sub>, or Fc fragments. Finally, a practical consideration arises from observations on the intracellular distribution of intact IgGs and fragments. The use of antibodies for immediate inhibition of intranuclear processes will require direct microneedle injection into nuclei, or one will have to use Fab or possibly Fab<sub>2</sub> fragments.

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