DEGRADATION AND LOCALIZATION OF IgG INJECTED INTO FRIEND ERYTHROLEUKEMIC CELLS BY FUSION WITH ERYTHROCYTE GHOSTS

Moshe WASSERMAN, Richard G. KULKA and Abraham LOYTER
Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

Received 18 August 1977
Revised version received 2 September 1977

1. Introduction

Macromolecules have been microinjected into animal cells by first trapping them in human erythrocyte ghosts and then fusing the loaded ghosts to recipient cells with the aid of Sendai virus [1–3]. This method, referred to in our laboratory as 'ultra-microinjection', has been used to inject ferritin [3], bovine serum albumin (BSA) [2,4], enzymes [2,5], IgG [6–8], tRNA [9], and globin messenger RNA [10] into cells. Previously we showed that about 10^6 molecules of ^{125}I-labelled BSA could be injected per hepatoma tissue culture cell if La^{3+} was added to promote fusion and prevent hemolysis [4]. Protein synthesis of the injected cells was inhibited under these conditions implying an impairment of viability [4]. Thus, although macromolecules could be efficiently microinjected in this way, the method seemed unsuitable for stuyding problems which require prolonged survival of recipient cells. Recently we showed that Friend erythroleukemic cells can be injected by the erythrocyte ghost fusion method in the presence of Ca^{2+} with complete retention of viability [8]. This opens the way to the study of problems related to the fate of the injected proteins within the cell. One of the most promising applications of the erythrocyte ghost injection method is the investigation of the mechanism of protein degradation in animal cells. By this method it is possible for the first time to inject a specific labeled protein into unlabeled cells and to characterize its breakdown products, including those which have no immunological or enzymatic activity.

In the present investigation we studied the intracellular distribution and rate of degradation of ^{125}I-labelled IgG injected into Friend erythroleukemic cells. Most of the injected IgG was found to be strongly bound to the nuclei. The degradation of IgG was slow and occurred in two phases.

2. Materials and methods

2.1. *Cells*

Human blood type O Rh positive, aged 3 weeks, was used. The blood was centrifuged for 5 min at 650 × *g*. The serum and the buffy layer containing white cells were discarded. The erythrocytes were washed 3 times with a solution containing 160 mM KCl and 20 mM Tricine–NaOH buffer, pH 7.4 (solution K^+).

Friend erythroleukemic cells, clone 745, were grown in Dulbecco's modified Eagle medium plus 10% calf serum. The cells were washed once with a solution containing 160 mM NaCl and 20 mM Tricine–NaOH buffer, pH 7.4 (solution Na^+), and resuspended in the same solution to a density of 2×10^7 cells/ml.

2.2. *Sendai virus*

Sendai virus was isolated and its hemagglutinin titre was determined as described previously [11]. The virus was inactivated by exposure to ultraviolet light as described by Harris et al. [12].

2.3. *Preparation of human erythrocyte ghosts*

An erythrocyte suspension of 35% (v/v) containing 0.5 mg/ml cytochrome *c* and 1 mg/ml ^{125}I-labelled IgG ($2–5 \times 10^7$ cpm) was dialyzed for 3 h against

North-Holland Publishing Company – Amsterdam

1000 vol. solution containing 40 mM KCl, 10 mM Tricine–NaOH buffer, pH 7.4. At the end of the dialysis, 0.05 vol. solution containing 2.2 M KCl and 0.02 M $MgSO_4$ were added, and the suspension was incubated for 30 min at 37°C. The ghosts were collected by centrifuging for 15 min at 12 000 × *g*. The pellet was washed twice with solution K^+ containing 0.5 mg/ml bovine IgG and the pellet was suspended with solution K^+ to give a concentration corresponding to 12.5% of original erythrocytes.

2.4. *Iodination of bovine IgG*

Bovine IgG (Sigma Chemical Co.) was purified by means of a DEAE cellulose (DE.52 Whatman) column [13] and iodinated as described before for BSA [4].

2.5. *Fusion of Friend cells with erythrocyte ghosts*

Fusion was performed as described before [8]. After fusion, cells were washed 3 times with solution Na^+, layered over a 10 ml cushion of 50% (v/v) calf serum in Dulbecco's medium and centrifuged at 180 × *g*. Cells were suspended in growth medium and samples were taken as required.

2.6. *Disruption of cells and distribution of IgG in fractions*

Cells were washed and resuspended in solution Na^+ containing 1% Triton X-100 and 5 mM Mg^{2+}. Nuclei were collected by centrifuging at 12 000 × *g* for 10 min or by centrifuging at 50 000 × *g* for 1 h over a 2 M sucrose, 3 mM $CaCl_2$, 10 mM Tris–HCl buffer, pH 7.8, cushion [14].

3. Results

Table 1 shows that, using the erythrocyte ghost injection technique, about 2×10^5 molecules of IgG can be injected per Friend erythroleukemic cell when about 6×10^4 molecules are trapped in one erythrocyte ghost. Thus, on the average, the contents of 3 ghosts were injected per cell.

When free IgG was added to the medium during fusion of unfilled ghosts with Friend cells, a significant number of molecules of IgG were introduced into the cells (about 7% of the number introduced when the same amount of IgG was inside the ghosts) (table 1). Thus, erythrocyte ghost-mediated injection of IgG was about 15-times more efficient than the uptake of IgG from the medium under fusion conditions.

Figure 1a shows the time course of degradation of injected IgG. The criterion of degradation was the appearance of trichloroacetic acid soluble ^{125}I. The degradation of IgG was biphasic consisting of an initial rapid phase lasting 4 h followed by a slow, linear phase which continued for at least 40 h. During the first 4 h the rate of breakdown was about 2%/h, and during the subsequent period it was about 0.4%/h. A possible reason for the transient rapid degradation of IgG was that fusion temporarily accelerated intracellular protein turnover. In order to test this, we compared the time course of degradation of endogenous cellular proteins in fused and unfused cells previously labeled by incubation with [^{3}H]leucine. Figure 1b shows that both in fused and unfused cells the degradation of protein is biphasic consisting of an initial phase of rapid degradation followed by a slower phase. Fusion of cells with ghosts did not significantly alter the rate of protein degradation. The initial rate of degradation of endogenous protein was about 3%/h and, after longer incubation, it decreased to about 1%/h. Thus, after 40 h 60% of the endogenous protein was degraded, whereas only 22% of the IgG was degraded during the same interval.

Table 1
Efficiency of injection of ^{125}I-labelled IgG into Friend erythroleukemic cells

Number of ^{125}I-labelled IgG molecules trapped per ghost	% Fusion	Number of ^{125}I-labelled IgG molecules in fusion system	Number of molecules injected/cell	Relative % injection
6×10^4	80	1.7×10^{14}	1.8×10^5	100
0	80	4.2×10^{14}	1.2×10^4	6.7

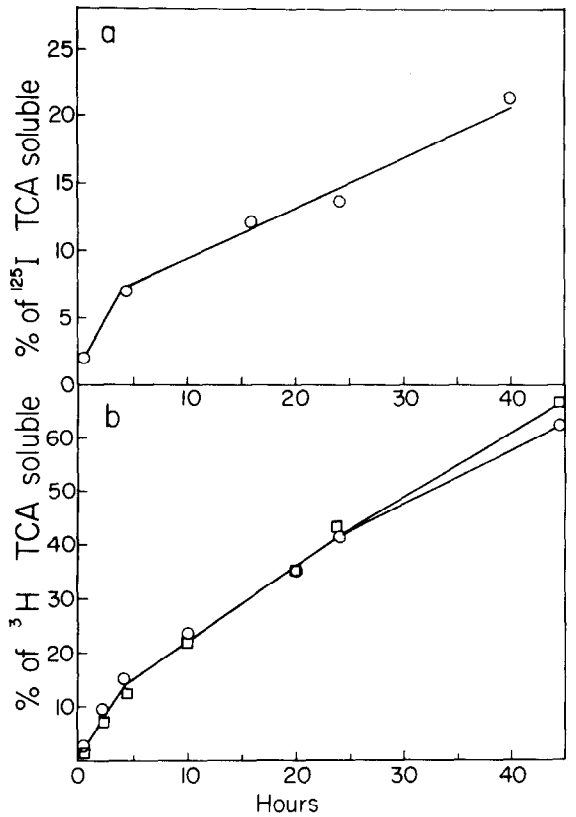

Fig.1. (a) Kinetics of degradation of ^{125}I-labelled IgG microinjected into Friend cells. (b) Kinetics of degradation of endogenous protein of fused (o——o) and unfused (□——□) Friend cells. Cells were incubated for 24 h in Dulbecco's medium containing 3 μCi/ml [^{3}H]leucine. Before fusion cells were washed once with fresh medium and twice with solution Na^{+} and resuspended in the same solution to a density of 2 × 10^{7} cells/ml.

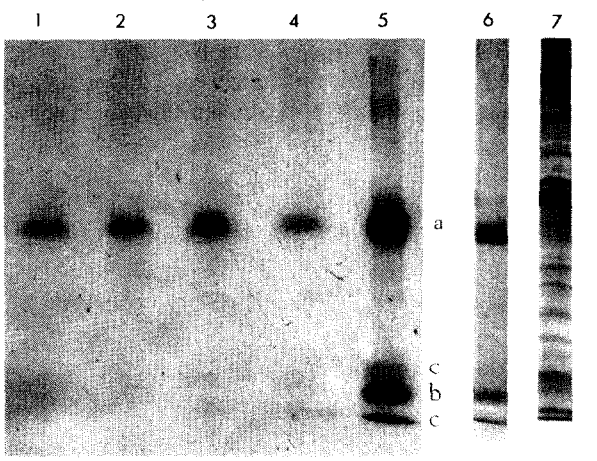

Fig.2. Autoradiograms of slab gels after electrophoresis of extracts of Friend cells injected with ^{125}I-labelled IgG. After fusion with erythrocytes loaded with ^{125}I-labelled IgG, 2 × 10^{7} cells containing 60 000 cpm were incubated at 37°C. At intervals samples of 2 × 10^{6} cells were removed, the cells were sedimented and resuspended in 0.1 ml solvent buffer ([17] method 1). The extracts were subject to electrophoresis on SDS–polyacrylamide slab gels containing 15% acrylamide [17]. After electrophoresis the slab gels were stained with Coomassie blue and dried. X-ray film (Eastman Kodak Company) was exposed to the dried gel for 21 days. Columns 1–4 are autoradiograms of radioactive protein peaks of extracts of cells incubated for 0, 1, 20 and 44 h, respectively. Column 5 is the autoradiographic pattern of the injected IgG. Column 6 is a Coomassie blue pattern of IgG. Column 7 is a Coomassie blue pattern of an extract of Friend cells that were microinjected with ^{125}I-labelled IgG. Band (a) IgG heavy chain; Band (b) IgG light chain; Bands (c) contaminating proteins.

Figure 2 shows autoradiograms of extracts of cells injected with ^{125}I-labelled IgG and disrupted after different times of incubation. At all times of incubation the same bands of radioactivity were found in the extracts as were present in the starting material. These were IgG heavy chain (a), IgG light chain (b) and traces of other material (c). Thus, most of the injected IgG, which was not degraded, was recovered in a form having the same molecular weight as light and heavy chains.

When cells were disrupted, 80–90% of the injected IgG was found to be tightly bound to the nuclear fraction (table 2). The same results were obtained when the cells were disrupted by freezing and thawing, French press (not shown), 1% Triton X-100, or by a mixture of 1% Triton X-100 plus 2% sodium deoxycholate. NaCl up to 1.5 M or excess unlabeled IgG in the disruption medium did not decrease the binding of the IgG to the nuclear fraction. Treatment of the nuclei with 2% sodium dodecyl sulfate released the ^{125}I-labelled IgG into the soluble fraction. The IgG remained bound to the nuclei during purification by centrifuging through a cushion of 2 M sucrose (table 2, Exp. 2). An increase of 20-fold in the number of IgG molecules injected per cell did not affect the relative distribution of IgG between the nuclear and soluble fractions (table 2, exp. 3). Binding of IgG to the nucleus is apparently a rapid process since it was

Table 2
Binding of injected ^{125}I-labelled IgG to the nuclear fraction of Friend erythroleukemic cells

Experiment number	Disruption method	Other treatment	Separation method	% in nuclear fraction
1[a]	Triton X-100 1%	None	Centrifugation at 12 000 × *g*	92
		Sodium deoxycholate 2%		89
		Sodium dodecylsulphate 2%		19
		Bovine IgG 20 mg/ml		94
		NaCl 1.5 M		94
2	Triton X-100 1%	None	Centrifugation through	82
	Freezing and thawing	None	2 M sucrose cushion	79
3	Triton X-100 %	2.8×10^5 IgG molecules/cell[b]	Centrifugation at 12 000 × *g*	97
		6.5×10^6 IgG molecules/cell		97
4	Triton X-100 1%	None	Centrifugation at 12 000 × *g*	85
	Enucleation with[c] cytochalasin B	None	Centrifugation through Ficoll gradient	87[d]

[a] Additions were present in the medium during disruption of the cells
[b] In this experiment the average number of IgG molecules inside each cell at the time of disruption was $1 \times (2.8 \times 10^5)$ or $20 \times (6.5 \times 10^6)$ the approximate number present in other experiments
[c] Enucleation of fused cells was performed with cytochalasin B and cytoplasts and karyoplasts were isolated according to Wigler and Weinstein [16]
[d] % of ^{125}I in karyoplasts

maximal even when the cells were disrupted immediately after fusion (which took 30 min). Experiment 4 in table 2 shows that when cells were enucleated with cytochalasin B, 85% of the injected ^{125}I-labelled IgG was associated with the karyoplasts while very little ^{125}I-labelled IgG was found in the cytoplast fraction (not shown).

4. Discussion

The erythrocyte ghost-mediated microinjection method is a powerful tool for following the intracellular localization and rates of breakdown of specific proteins. It makes it possible to inject labeled proteins into unlabeled cells and to detect their breakdown products. Pathways of degradation of specific proteins can be followed even when they have lost all enzymatic and/or immune activity.

Here we studied the fate of exogenous IgG in Friend cells which do not normally contain IgG. Although the initial burst of fast breakdown of IgG was the same as that of endogenous protein, the subsequent rate of breakdown of IgG was much slower than the endogenous rate. It is surprising that IgG, which presumably consists of a uniform population of molecules, is broken down in two phases, and we cannot suggest a satisfactory explanation for this. The similar biphasic kinetics of endogenous protein is explained much more easily by the fact that some of the cell proteins turn over more rapidly than the others [15].

^{125}I-labelled IgG is bound tightly to the nuclei in a manner which is not easily reversed by detergents or high salt concentrations. The labeled IgG does not readily exchange with unlabeled IgG. Since in the enucleation experiments almost all of the IgG is found in the karyoplast fraction it seems that the association of IgG with the nuclear fraction is not due to adsorption of IgG to nuclei during disruption. We did not succeed in saturating all the nuclear binding sites for IgG which must number at least 7×10^6. It is not clear whether tight binding to nuclei is characteristic only of IgG or will be found to be a property of other

injected proteins. Perhaps the slow turnover of IgG is related to its binding to the nucleus.

Acknowledgements

We thank Mrs Rachel Ampel for skilled and devoted technical assistance. This work was supported by grants to A. L. from the US-Israel Binational Science Foundation and the Israel Cancer Association.

References

[1] Furusawa, M., Nishimura, T., Yamaizumi, M. and Okada, Y. (1974) Nature 249, 449–450.
[2] Schlegel, R. A. and Rechsteiner, M. C. (1975) Cell 5, 371–379.
[3] Loyter, A., Zakai, N. and Kulka, R. G. (1975) J. Cell Biol. 66, 292–304.
[4] Wasserman, M., Zakai, N., Loyter, A. and Kulka, R. G. (1976) Cell 7, 551–556.
[5] Wille, W. and Willecke, K. (1976) FEBS Lett. 65, 59–62.
[6] Nishimura, I., Furusawa, M., Yamaizumi, M. and Okada, Y. (1976) Cell Struc. Func. 1, 197–201.
[7] Rechsteiner, M. (1976) IIIrd Decennial Rev. Conf. Cell Tissue and Organ Culture, Lake Placid, New York.
[8] Wasserman, M., Loyter, A. and Kulka, R. G. (1977) J. Cell Sci. (in press).
[9] Kaltoft, K., Zeuthen, J., Engback, F., Piper, P. W. and Celis, E. J. (1976) Proc. Natl. Acad. Sci. USA 73, 2793–2797.
[10] Anderson, W. F. and Krueger, L. J. (1976) J. Cell Biol. 70, 185a.
[11] Toister, Z. and Loyter, A. (1970) Biochem. Biophys. Res. Commun. 41, 1523–1530.
[12] Harris, H., Watkins, T. F., Ford, C. E. and Schoefl, G. J. (1966) J. Cell Sci. 1, 1–30.
[13] Nowotny, A. (1969) in: Basic Exercises in Immunochemistry, pp. 10–12, Springer–Verlag, New York.
[14] Chauveau, J., Moule, Y. and Ruiller, C. (1956) Exptl. Cell Res. 11, 317–321.
[15] Epstein, D., Elias-Bishko, S. and Hershko, A. (1975) Biochemistry 14, 5199–5204.
[16] Wigler, M. H. and Weinstein, B. I. (1975) Biochem. Biophys. Res. Commun. 63, 669–674.
[17] Weber, K. and Osborn, M. (1975) in: The Proteins, 3rd edn. (Neurath, H. and Hill, R. C. eds) Vol. 1, pp. 179–223, Academic Press, New York.