Russell Bodies: A General Response of Secretory Cells to Synthesis of a Mutant Immunoglobulin Which Can Neither Exit from, Nor Be Degraded in, the Endoplasmic Reticulum

Caterina Valetti,* Carlo E. Grossi,† César Milstein,§ and Roberto Sità††

*Istituto Scientifico San Raffaele, 20132 Milano, Italy; †Istituto Nazionale per la Ricerca sul Cancro, 16132 Genova, Italy; and §Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, UK

Abstract. Dilated cisternae of the ER resembling Russell Bodies (RBs) are induced in light (L) chain producing myeloma cell lines by transfection of a jA heavy (H) chain gene lacking the first constant domain (μΔCH1). RBs do not appear to be tissue specific, since they are also induced in a rat glioma cell line transfected with pACHI and L chain genes.

Efficient RB biogenesis requires H-L assembly and polymerization. The mutant Ig is partially degraded in a pre-Golgicompartment. The remnant, however, becomes an insoluble lattice when intersubunit disulphide bonds are formed. The resulting insoluble aggregate accumulates in RBs. Replacing the COOH-terminal cysteine of μΔCH1 chains with alanine reverses the RB-phenotype: the double mutant μDeltaΔCH1 chains assemble noncovalently with L and are secreted as H2L2 complexes. Similarly, secretion of μΔCH1 chains can be induced by culturing transfecant cells in the presence of reducing agents. The presence of RBs does not alter transport of other secretory or membrane molecules, nor does it affect cell division. Resident proteins of the ER and other secretory proteins are not concentrated in RBs, implying sorting at the ER level. Sorting could be the result of the specific molecular structure of the insoluble lattice. We propose that RBs represent a general response of the cell to the accumulation of abundant, nondegradable protein(s) that fail to exit from the ER.

One hundred years ago, Russell described certain intracellular bodies in marginal areas of tumours, which he thought were fungi representing the etiological agent of cancer (Russell, 1890). The origin of these structures, which were later referred to as Russell bodies (RBs), has been the subject of considerable research and controversy, over many years. They were thought by some to be pathological secretions or aggregates of normal secretion, or even secretory granules, while others proposed that they resulted from degeneration of plasma cells, from abortive differentiation of haemocytoblasts, or due to red blood corpuscles taken up by plasma cells. Pearse, in 1949, showed that they contained mucoproteins secreted by plasma cells (Pearse, 1949). Others confirmed these observations, and further showed the involvement of γ-globulins and antibodies (reviewed by Maldonado et al., 1966).

The origin of RBs has remained controversial. The involvement of the ER and a similarity between RBs and pancreatic secretory granules were suggested by Maldonado et al. (1966), who also suspected RBs to be similar in character to those described by Mott (1905). It is now generally agreed that “Mott” cells (or Morula cells) and “Russell” cells are essentially similar. The nature of this type of cells has remained under scrutiny. Interest revived with the observation that the “Mott phenotype” could be found in certain cell lines (Lockhorst et al., 1987) and also in hybridomas (Weiss et al., 1984; Alanen et al., 1985). The possibility that they are intermediate stages of normal secretion is no longer tenable, because hybridoma cells with RBs fail to secrete, or secrete very little Ig (Weiss et al., 1984; Alanen et al., 1985). The question which has been increasingly raised among those who considered RBs to be abnormal structures, is why they occur at all. The abnormality could originate from defects in the secretory apparatus, as suggested by Alanen et al. (1987). In this paper, we show that RBs can be generated by introducing abnormal Ig genes into otherwise healthy myeloma cell lines, and even in nonlymphoid secretory cells. We propose that RBs originate as an SOS compartment, where abnormal proteins which cannot be secreted, but have escaped intracellular degradation, can be accumulated without blocking the normal secretory pathway. These results imply the existence of subcompartments within the ER.

Materials and Methods

Cells, Plasmids, and Transfections

J558L (Qi et al., 1983), NSO (Cowan et al., 1974; Galfre and Milstein,
1981) C6 and C6A (Cattaneo and Neuberger, 1987) were maintained in DME supplemented with 1 mM glutamine, penicillin, streptomycin, and 10% FCS, and were transfected by electroporation (Potter et al., 1984).

Plasmid pSVVpAACHI (see Fig. 1), obtained by deleting the SacI fragment located between the enhancer region and the Cpg2 from pSVVpA1 (Neuberger, 1983), was a kind gift of Dr. M. S. Neuberger (MRC Laboratory of Molecular Biology, Cambridge, MA). To achieve Ig expression in nonlymphoid cells, the endogenous Ig promoter was substituted with the heat-inducible hsp70 promoter (Pelham, 1982). Thus, the exon encoding the first constant domain was deleted from pSVV-HSVp1 (a gift of Dr. J. Mason University of California at San Francisco, [Mason et al., 1988]) to generate pSVV-HSVpμΔCtn. To construct pSVV-HSVpμΔCtn, the BglII-SacI vector fragment of pSVV-HSVpΔCtn was ligated to the Cpg2-4 fragment of pSVV-[In 1,3,4,5]aia obtained by digestion with BglII (complete) and SacI (partial [Sittia et al., 1990]). pSVV was used to transfect NSO cells, while pSVV-HSA1 was employed to generate nonlymphoid transfectants (Cattaneo and Neuberger, 1987). All heavy chain encoding plasmids carry the gpt gene, while those encoding light chains carry the neo gene.

Stable transfectants were selected in medium containing 5 μg/ml mycophenolic acid, 13.6 μg/ml hypoxanthine, and 0.25 mg/ml xanthine (all from Sigma Chemical Co., St. Louis, MO) for pSV2gpt, and in 1 μg/ml G418 (Gibco Laboratories, Grand Island, NY) for pSV2neo-based vectors. Positive clones were selected by immunofluorescence with class-specific antibodies as previously described (Sittia et al., 1987).

Biosynthetic Labeling, Immunoprecipitation, and Gel Electrophoresis

Cells were washed twice in methionine-free MEM and cultured for 2-18 h at 37°C in the same medium supplemented with 100-200 μCi/ml of [35S]methionine (specific activity >800 Ci/mmol, Amersham, Milano, Italy). 2% dialyzed FCS, antibiotics, and 1 mM glutamine. Pulse-chase experiments were performed as previously described (Sittia et al., 1987, 1990). For sugar labeling, cells were cultured for 1 or 2 h in glucose-free DME supplemented with 2% dialyzed FCS and either 50 mM glucose (200 μCi/mM) or 0.1% mannose (50 μCi/ml, both from Amersham). Cells were then washed once in PBS and lysed in NP-40 (0.25%) unless otherwise indicated.

Lysates and supernatants of labeled cells were precleared with protein A-Sepharose (Pharmacia, Uppsala, Sweden) and immunoprecipitated with pure rabbit anti-mouse (RAM)-μ or monoclonal antiidiotypic antibodies Ac 38 (Reth et al., 1979) followed by protein A-Sepharose, or with sepharose-bound (4 hydroxy 3 nitrophenyl) acetyl (NP) (Reth et al., 1979; Neuberger, 1983). A lysate and postfixed for 1 h at 37°C in the dark. The substrates for the localization of acid phosphatase and β-glucuronidase were prepared as previously described (Zicca et al., 1978; Ferrari et al., 1980; Landay et al., 1984). For the ultrastructural studies, fixed cells were rinsed in cacodylate buffer, postfixed for 1 h with 1% osmium tetroxide in the same buffer, dehydrated through a graded ethanol series, and embedded in Spurr’s medium (Spurr, 1969). Thin sections were stained with uranyl acetate and lead citrate and analyzed with a Philips 301 electron microscope.

Results

Induction of RB in Plasmacytomas Transfected with a Defective Heavy Chain

Plasmid pSVV-μΔCtn (Fig. 1) encodes a μ chain that lacks the first constant domain (Ch1). When this plasmid is transfected into the mouse myeloma J558L, which produces and secretes λ L chains, the transfected cell line (J[AACHI], see Table 1), accumulates in its cytoplasm prominent RB-like structures (Fig. 2). Immunolocalization experiments showed that the defective H chain is primarily localized within these large vacuoles (Fig. 2, a and c). The structures are generally large and few in number (on a per cell basis), but cells with more than a dozen smaller vesicles are also detected. These structures are not found in J[μ] cells, a J558L line harboring plasmid pSVV-μ (Neuberger, 1983), which encodes wild type [wt]-μ (Sittia et al., 1987, 1990) or in other transfectants of J558L produced in our laboratory (not shown). The

Western Blotting

Polyacrylamide gels were transferred electrophoretically to nitrocellulose paper (Schleier & Schuell, Dassel, West Germany). Filters were incubated for 1 h in PBS containing 5% milk powder (Gesarl, Milan, Italy) and rinsed in PBS containing 0.05% Tween 20. To simultaneously detect heavy (H) and light (L) chains, filters were cut horizontally in correspondence of the pre-stained ovalbumin marker (46 kD), and the upper part, containing heavy chains, was incubated with alkaline phosphatase–conjugated rabbit anti-μ mouse-μ (Zymed, San Francisco, CA) and developed according to the manufacturer’s instructions. A biotinylated goat anti-μ antibody, followed by peroxidase-streptavidin (both from Amersham), was used for the lower part of the filters, to detect L chains. A monoclonal rat anti-mouse BiP (Bole et al., 1986) was employed, followed by peroxidase-conjugated rabbit anti-IgG (DakoPatts, Glostrup, Denmark).

Immunofluorescence

Immunofluorescence was performed on poly-L-lysine–coated slides as described previously (Sittia et al., 1987). Fluoresceinated and rhodaminated goat anti-mouse-μ and goat anti-μ mouse-λ were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Rat anti-mouse BiP (Bole et al., 1986) a gift of Dr. L. Hendriksen, Memphis, TN) rabbit anti-mouse endoplasmin (a gift of Dr. G. Koch, Cambridge, UK) and rabbit anti-protein disulphide isomerase (from Dr. R. Freedman, Canterbury, UK) were used in fixed immunofluorescence followed by fluorescein-conjugated goat anti-rabbit or goat anti-rabbit Ig (Cappell, Cochransville, PA). Fluoresceinated wheat germ agglutinin, a marker of the exocytic compartment downstream the Golgi apparatus (Tartakoff and Vassalli, 1983), was purchased from Sigma Chemical Co. and used according to the manufacturer instructions. Samples were inspected by either conventional or laser confocal microscopy (Leica CLSM, courtesy of A. D. Mian, Institute of Anatomy, University of Milan, Italy).

Cytochemistry and EM

Cells were fixed in suspension with 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6, for 30 min at room temperature. For cytochemical staining, fixed cells were washed with PBS, cytostreptinated onto slides, and incubated with the substrates for 1 h at 37°C in the dark. The substrates for the localization of acid phosphatase and β-glucuronidase were prepared as previously described (Zicca et al., 1982; Ferrari et al., 1980; Landay et al., 1984). For the ultrastructural studies, fixed cells were rinsed in cacodylate buffer, postfixed for 1 h with 1% osmium tetroxide in the same buffer, dehydrated through a graded ethanol series, and embedded in Spurr’s medium (Spurr, 1969). Thin sections were stained with uranyl acetate and lead citrate and analyzed with a Philips 301 electron microscope.

Figure 1. Structure of plasmids. The heavy chain–encoding plasmids are outlined. Construction of pSVV-μ was described by Neuberger (1983). In pSVV-HSVp1 (Mason et al., 1988) the endogenous Ig promoter (white box) was replaced with the hsp70 promoter (grey box). Deletion of the first constant domain (Ch1) was achieved by digestion with SacI and religation. The COOH-terminal cysteine was substituted with alanine by site-directed mutagenesis (Sittia et al., 1990). For other transfectants of J558L produced in our laboratory (not shown). The
vacular structures stain prominently also with anti-λ. However, unlike anti-μ, anti-λ stains brightly also other regions of the cytoplasm, corresponding to the ER and the Golgi apparatus (Fig. 2b). Thus, in J[μΔCH1] cells, mutant μ chains are concentrated within RBs, but only a fraction of the L chains is diverted to them (see below). When the plasmid pSVVμCH1 is used to transfect NSO myeloma cells, which do not express L chains, few if any RBs are detected in the transfectant (Fig. 2c). The occasional vacuoles detected are much smaller than those found in J[μΔCH1]. Su-
pervacuolestion of N[μΔCH1] with pSVVλ induces the for-
mation of vacuoles (Fig. 2d). Thus, the vacuole phenotype is not specific for one particular myeloma cell and is dependent on the protein structure, since L chains are essential.

At the EM level, the vacuoles appear to be delimited by a membrane covered by ribosomes, separated from a dense mass of material by a clear rim which excludes the electron dense dye (Fig. 3). Many cells containing vacuoles are mitotic (Fig. 3c), indicating that the presence of these extremely dilated cisterna does not affect cell division. This is consistent with the observation of a similar doubling time in J[μΔCH1] and J[μ1] cells.

The presence of ribsomes on the cytosolic face of the membrane (see Fig. 3e for a detail), suggests a relationship between the vacuoles and the ER. However, resident ER proteins are not concentrated in the vacuoles. Thus, antibodies against three such proteins, BiP (Fig. 2d), protein disulphide isomerase, and endoplasm (not shown), stained the vacuoles rather weakly (see also below). On the other hand, the staining pattern with fluoresceinated wheat germ agglutinin is indistinguishable in J[μΔCH1] and J[μ1] cells (Fig. 2, g and h, respectively), and more specifically we do not detect significant RB staining, suggesting that the protein has not traversed the Golgi apparatus.

RBs are often contiguous to normal ER stacks. Microscopical examination, however, does not reveal continuity between the two structures in myeloma transfectants. In addition, no fusion elements between endolysosomal vesicles and RBs are observed by EM or immunocytochemistry (data not shown).

**Defective Secretion of Mutated Ig**

The secretion of H and L chains differs considerably when the mutant J[μΔCH1] and the wild type J[μ1] transfectants are compared (Fig. 4a). Very little mutant μ chain is precipi-
tated from the supernatants of pulsed J[μΔCH1] cells even after prolonged chase periods (data not shown). The μΔCH1 chains present in the supernatant have a slower mobility on SDS-PAGE than the intracellular material, suggesting residual secretion rather than cell death. As for the L chains, it should be remembered that in secretory B cells L chains are present in large excess with respect to μ chains, and this may be even more marked in H chain transfectants. In the case of both J[μΔCH1] and J[μ1] the excess of L chains is at least 3 fold, and probably much larger (data not shown). The excess (free) L chains are secreted by J[μΔCH1] with kinetics similar to the control transfectants without RBs (Fig. 4a).

Therefore, the presence of RBs is compatible with protein secretion.

To further compare biosynthesis and secretion of mutant and wild type μ chains, equal numbers of J[μΔCH1] and J[μ1] cells were mixed, labeled for 2 h with [35S]methionine, [14C]mannose, or [3H]galactose, and immunoprecipitated with anti-μ (Fig. 4b). Under these conditions, no [35S]me-thionine–labeled mutant H chain is detectable in the supernatant (Fig. 4b, lane 2). Less galactose is incorporated into μΔCH1 chains, as compared to wild type μ (Fig. 4b, lanes 5 and 6), but traces of secreted material can be detected in this way. The [14C]mannose/[%S]methionine ratio is approximately twice as high in J[μΔCH1] than in J[μ1], suggesting either increased incorporation or reduced trimming of manno-

Table 1. Properties of Cell Lines and Transfectants

| Cell line-Plasmid                  | Cell type           | Abbreviation |
|-----------------------------------|---------------------|--------------|
| J558L [pSV-Vμ1]                   | mouse myeloma       | J[μ1]        |
| J555L [pSV-VμΔCH1]                |                     | J[μΔCH1]     |
| J555L [pSV-VμΔCH1]                |                     | J[μμΔCH1]    |
| NSO [pSV-VμΔCH1]                  | mouse myeloma       | N[μΔCH1]     |
| NSO [pSV-VμΔCH1 + pSV-Vλ1]        |                     | N[μCH1-λ]    |
| C6 [pSV-HSVμΔ + pSV-HSVλ1]        | rat glioma†         | C6[μ1]       |
| C6 [pSV-HSVμΔCH1 + pSV-HSVλ1]     |                     | C6[μΔCH1]    |
| C6 [pSV-HSVμΔCH1 + pSV-HSVλ1]     |                     | C6[μμΔCH1]   |

* Indicates endogenous immunoglobulin chain production; both J558L and NSO produce endogenous J chain.
† C6 glioma cells do not produce H, L, or J chains (Cattaneo and Neuberger, 1987).

Assembly and Polymerization of Mutant IgM Are Essential for the Formation of RBs

The CH1 is the binding domain for both L chains and BiP (Bole et al., 1986; Hendershot et al., 1987). L chains are, however, capable of binding μΔCH1 chains through the VH domain in a noncovalent way. This is not the first example of a mutant Ig that lacks the CH1 domain but binds L chains (Secher et al., 1977). Under nonreducing conditions, the μΔCH1 bands comigrate in J[μΔCH1] and N[μΔCH1] excluding covalent interactions with λ (results not shown). That the deletion of CH1 does not affect VH-Vt pairing is shown by...
Figure 2. Structures resembling RB are present in J[μΔCH1] cells. Confocal immunofluorescence analysis of myeloma transfectants. (a, b, c, d, and g) J[μΔCH1]; (e) N[μΔCH1]; (f) N[μΔCH1-λ]; (h) J[μ1] cells. Slides were stained with fluoresceinated or rhodaminated goat anti-mouse-anti-μ (a, e, f, and c, respectively). a and b show the same cell stained simultaneously with fluoresceinated anti-μ (a) and rhodaminated goat anti-mouse-λ (b). Similarly, c and d show the same cell costained with rhodaminated anti-μ (c) and with rat anti-BiP followed by fluoresceinated goat anti-rat Ig (d). BiP appears to be largely excluded from the vacuoles (see arrows). In g and h, J[μΔCH1] and J[μ1] cells were stained with rhodaminated WGA. Bar, 5 μm.
precipitation of intracellular material with either the antigen (NP-Sepharose, Fig. 5 a, lane 2) or the antidiotypic antibody Ac38 (results not shown), both of which require Vh-Vl interaction (Reth et al., 1979). However, precipitation with anti-μ antiserum does not coprecipitate much L chain, which suggests that the interaction of L chain and μΔChI may be inhibited by the antiserum and/or that the noncovalent Vh-Vl association is stabilized by the antidiotypic antibodies and by hapten. The higher ratio of L and H chain precipitated by antigen (see legend to Fig. 5 a) (and also by antidiotype, not shown) suggests that the stoichiometry of the two chains is not the usual 1:1, but more likely 2:1 in favor of L chains. It remains to be seen whether this is a reflection of a real structural difference or an artifact due to variations in labeling resulting from different intracellular pools of unassembled chains.

The kinetics of accumulation of polymeric wild type and mutant IgM differ considerably. Thus, in the J[μI] transfectant, as in other IgM secreting cells, an equilibrium between ~20% polymeric and 80% non-polymeric IgM is reached after between one and two hours of chase, when newly synthesized polymers are being secreted (Sitia et al., 1987). In the mutant J[μΔChI], polymers are formed more slowly, and they continue to accumulate at the expense of preformed monomers (Fig. 5, b and c). Thus, after prolonged chase, labeled monomers are virtually undetectable, while polymers remain in abundance.

**Mutant IgM Accumulates in RBs in the Form of Stable Aggregates**

When the pellet of a lysate obtained with 0.25% NP-40 is extracted with 2% SDS, a considerable amount of μΔChI chains can be solubilized and detected by Western blotting (see Fig. 6 a, lane 6). L chains are also detected in the NP-40-insoluble fraction. Mutant IgM is solubilized also by urea (8M, lane 7) and, partially, by 2-mercaptoethanol (2ME, 700 mM, lane 9). Analysis under nonreducing conditions (Fig. 6, lanes 10 and 11), reveals that the IgM present in the insoluble material is largely in the polymeric form. On the contrary, in J[μI] (Fig. 6, lanes 1-3) as well as in other transfectants without RBs (not shown), virtually all Ig is found in the 0.25% NP-40-soluble fraction. Thus, part of μΔChI chains forms a precipitate that is held together by both covalent and noncovalent interactions. BiP (Fig. 6 b) and PDI (not shown) are found only in the soluble NP-40 fraction. Since resident proteins of the ER and excess light chains are excluded from the core precipitate containing mutant IgM, aggregation appears to be a specific self-assembly of a mutant Ig lattice. As shown by pulse-chase experiments (Fig. 6 c; see legend for experimental details), μΔChI chains are soluble in NP-40 immediately after synthesis and, in this form, subject to fast degradation (see Fig. 6 d). However, the undegraded remnant progressively accumulates within the insoluble fraction in the form of polymeric molecules. The precipitated material appears to be rather stable and after 21 h ~30% of the initial radioactivity is still detectable (Fig. 6 d). The slight reduction in intensity of the NP-40-insoluble band between 4.5 and 21 h of chase is matched by a proportional increase in the secreted material. This suggests that part of the precipitate may recycle into a soluble and secretable pool (see below). In the absence of L chains

---

*Figure 3. EM analysis of J[μΔChI] cells. Note a mitotic cell in b, and, at higher magnification (c), numerous ribosomes on the membrane (cytosolic face) of the vacuole (thick arrow). Thin arrow points to a stack of normal ER. Bars, 1 μm.*
Figure 4. The secretory block in J[μΔCH1] is selective for mutant H chains. (a) J[μ1] (lanes 1-4) and J[μΔCH1] (lanes 5-8) cells were washed thoroughly and cultured at 1 × 10⁶/ml in medium containing 1% FCS. At the indicated times, aliquots of the supernatants were harvested, concentrated fivefold, and analyzed by Western blotting with anti-μ (upper panel) and anti-λ (lower panel) antibodies. (b) Equal numbers of J[μ1] and J[μΔCH1] cells were mixed and labeled for 2 h with [35S]methionine (lanes 1 and 2), [14C]mannose (lanes 3 and 4) or [3H]galactose (lanes 5 and 6). Wild type μ and μΔCH1 chains differ in molecular weight, and can be easily identified on SDS-PAGE (see arrows on left hand margin). Cell lysates (c) and supernatants (s) were immunoprecipitated with anti-A and analyzed by SDS-PAGE under reducing conditions. The μ/μΔCH1 ratio (calculated by densitometric analyses of the autoradiogram) was 1.25 in the sample labeled with [35S]methionine (lane 1), and 0.65 when cells were labeled with [14C]mannose (lane 3).

(N[μΔCH1] cells) the mutant μ chain does not accumulate into an insoluble precipitate and is rapidly degraded (Fig. 6d). That the insoluble material is likely to correspond to the content of RBs is indicated by the identification of RB-like structures in the 0.25% NP-40 pellets by immunofluorescence (not shown).

Mutating the COOH-terminal Cysteine Restores Secretion of μΔCH1 Chains

The importance of polymers in the formation of RBs is demonstrated by the phenotype of another cell line prepared by transfecting J558L with a mutant of μΔCH1 in which Cys 575 was replaced by Ala. The mutation restores efficient secretion (Fig. 7a). NP-Sepharose precipitates μΔCH1 chains, indicating that the latter are associated with λ. Like in the case of μΔCH1, this association is noncovalent. The transfectant cell line, J[μΔCH1], contains few if any RBs, and anti-μ stains primarily the Golgi region (Fig. 7b). Therefore, the two phenotypic characteristics of the Mott phenotype, impaired secretion and presence of RBs, are reversed by the removal of the COOH-terminal sulfhydryl (SH) group. This SH group is responsible for both polymerization of IgM subunits and retention in the ER of assembly intermediates (Siti et al., 1990).

μΔCH1 Chains Are Secreted in the Presence of Reducing Agents

It is known that 2ME induces secretion of unassembled, non-S-S-bonded intermediates (Alberini et al., 1990). culturing J[μΔCH1] cells for 2 h in the presence of 2ME (7.1 mM) also induces secretion of μΔCH1 chains (Fig. 7c). Since secreted μΔCH1 chains underwent terminal glycosylation (as indicated by their slower electrophoretic mobility) the result cannot be explained by reduced cell viability in the presence of reducing agents (see also Alberini et al., 1990). Thus, as in wild type μ chains, the COOH-terminal cysteine plays an important role in retention of unpolymerized chains, but unlike wild type chains, polymerization does not lead to secretion or to fast degradation, but to accumulation of polymers in RBs, likely in the form of insoluble precipitates. Interestingly, although less efficiently than when added immediately after the chase, 2ME can induce secretion of μΔCH1 chains even after overnight chase (Fig. 7c), when virtually all mutant IgM is NP-40-insoluble within RB (Fig. 6c). Thus, RBs are at least functionally connected with the secretory apparatus, and part of their contents can either continuously recycle between the RBs and the cis-Golgi or escape from the RBs, join the exocytic pathway, and be secreted.

Russell Body-like Structures Are Also Induced by the Defective Gene in Glioma Cells

The importance of the protein itself in the genesis of RBs is further emphasized by the appearance of similar structures in nonlymphoid cell transfected with pSV-HSVμΔCH1. The rat glioma cell chosen was C6α which expresses a heat-inducible λ chain gene. In these cells, transfection with the wild type μ gene [μ1] allows expression and secretion of fully assembled IgM (Cattaneo and Neuberger, 1987). A similar construct was made, substituting the intact μ gene for μΔCH1 (driven by a heat-inducible promoter, see Fig. 1). Fig. 8a shows that in the C6α[μΔCH1] transfectant (but not in C6α[μ1], Fig. 8b) the defective H chain is prominently stained in intracellular structures. These structures are
Figure 5. μΔCH1 chains assemble noncovalently with L chains and form polymers. (a) Noncovalent assembly between μΔCH1 and light chains. Lysates of J[μΔCH1] and J[μ1] cells labeled for 3 h with [35S]methionine were immunoprecipitated with anti-μ antibodies and protein A–sepharose (anti-μ; lanes 3 and 4) or with sepharose-bound nitrophenacetyl (NP; lanes 1 and 2), and analyzed by SDS-PAGE under reducing conditions. The results with antiidiotypic antibodies (Ac38) were identical to those obtained with NP-Sepharose (not shown). In J[μΔCH1] cells, the H/L ratio, as calculated by densitometric analyses of the gel shown, was 44 in anti-μ precipitate (lane 4) and 1.7 in NP-sepharose precipitate (lane 2). By contrast, in J[μ1] cells the H/L ratio was 6.1 in both samples, as expected from a covalent interaction. (b and c) Rate of polymerization of mutant IgM. J[μΔCH1] cells were pulsed for 5 min with [35S]methionine and chased for indicated times before lysis and immunoprecipitation with anti-μ. Immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions (b). The rate of polymerization was calculated by densitometric analysis of the autoradiograms shown in b and compared to that of wild type μ (J[μ1] cells, data from Sitia et al., 1987). Data show the percentage of total intracellular radioactivity associated with polymers as a function of time (c). In J[μ1] cells, the plateau reflects the time at which the rate of polymerization of monomers (which are not secreted) and the rate of secretion of polymers reach an equilibrium. In J[μΔCH1], the ratio of polymers over monomers increases with time, because in this case polymers are not secreted.
Figure 6. \(\alpha\text{ACH1}\) chains form stable aggregates that accumulate in RBs. (a) Part of mutant IgM forms insoluble aggregates. Identical aliquots of \(J[\mu]\) and \(J[\alpha\text{ACH1}]\) cells were lysed with SDS or 0.25% NP-40 (all other lanes). The material soluble in SDS (lanes 1 and 4) or NP-40 (Sol, lanes 2 and 5) was run directly on SDS-PAGE, while the NP-40 pellets were extracted with 2% SDS (lanes 3, 6, and 11), 8 M urea (lane 7) or 2 ME (7.1 mM, lane 8 or 710 mM, lane 9). Lanes 10 and 11 were run on a 4% acrylamide gel under nonreducing conditions (Ziegler and Hengartner, 1977), and analyzed by western blotting with anti-\(\alpha\). Lanes 1-9 were run under reducing conditions on a 10% acrylamide gel and the blot was cut into two parts: the upper part was developed with anti-\(\mu\), the lower with anti-\(\lambda\) (see Materials and Methods). Prestained molecular weight markers (purchased from BioRad Italia, Segrate, Italy) were run to allow the identification of the bands as indicated by arrows. (b) ER resident proteins are excluded from the aggregates. Aliquots of \(J[\alpha\text{ACH1}]\) (lanes 1-3) and \(J[\mu]\) (lanes 4-6) were washed and lysed with SDS (lanes 1 and 6) or NP-40. The NP-40-soluble material (lanes 2 and 5) and the 8 M urea extracts from NP-40 pellets (lanes 3 and 4) were resolved by SDS-PAGE under reducing conditions. Western blots were developed with rat anti-mouse BiP (upper panel), or anti-\(\mu\) antibodies (lower panel). (c) Kinetics of accumulation of \(\alpha\text{ACH1}\) chains into RBs. At the indicated chase times, \(J[\alpha\text{ACH1}]\) cells pulsed for 30 min with \([\text{35S}]\)methionine were lysed in 0.25% NP-40. Both NP-40-soluble material (sol) and the 8 M urea extract from pellets (pel, diluted 10-fold in PBS before immunoprecipitation), as well as the supernatants were immunoprecipitated with anti-\(\mu\) and analyzed by SDS-PAGE and densitometry. The distribution of total recovered radioactivity between the different fractions (%) is shown at each point of chase. For total recovery, see data on d. (d) Kinetics of degradation of \(\alpha\text{ACH1}\) chains in J558L or NSO transfectants. The intracellular turnover of \(\alpha\text{ACH1}\) chains was determined by pulse–chase experiments of \(J[\alpha\text{ACH1}]\) and \(N[\alpha\text{ACH1}]\) followed by immunoprecipitation, SDS-PAGE, and densitometry. The results are expressed as percent radioactivity relative to 0 chase. Data represent the mean of two experiments.

Discussion

Biogenesis of Russell Bodies

Our data demonstrate that the synthesis of a mutated Ig (but smaller but more abundant than the ones formed in J558L, and in this sense more like classic RBs. When analyzed by EM, these structures show clear similarities with the vacuoles found in \(J[\alpha\text{ACH1}]\) (Fig. 8). Since C6 glioma cells do not synthesize endogenous J chains (Cattaneo and Neuberger, 1987), the latter are not necessary for RB formation. Removal of the COOH-terminal SH restores secretion of \(\alpha\text{ACH1}\) chains also in glioma cells (Fig. 8). Similarly, the presence of RB-like structures in C6 cells does not inhibit normal secretion (e.g., of endogenous fibronectin; Fig. 8, upper arrow).

Since the transfected \(\alpha\text{ACH1}\) gene is driven by an inducible (heat shock) promoter, it was possible in this case to show that the formation of the RB-like vesicles was induced with the expected kinetics of accumulation of \(\alpha\text{ACH1}\) (Fig. 8, and data not shown). Finally, formation of RB-like structures was largely prevented if heat-shocked C6\(J[\alpha\text{ACH1}]\) were cultured in the presence of 2 ME (data not shown).
not of the wild type counterpart) is sufficient to induce the formation of intracellular structures that share all the features of RBs. These structures were induced in the course of several independent transfections of J558L myeloma cells, which, as indicated by their capability of secreting endogenous λ chains and other transfected genes, have a functional secretory apparatus. The importance of the protein structure (as opposed to the cell phenotype, [Alanen et al., 1987]) in the induction of RBs, is underscored by the observation that transfection of the same mutant Ig into another myeloma line or into a nonlymphoid cell line also induces a similar phenotype. The morphology of the bodies was not identical. Paradoxically, the glioma transfectants contained the most commonly observed RB morphology, while in the myelomas RBs were larger and fewer in number (on a per cell basis).

Why do RBs form? There are several myelomas producing nonsecreted Ig chains or mutants that do not have the Mott phenotype (e.g., NS1 [Cowan et al., 1974; Galfré and Milstein, 1981], N[μl], J[y2b-μtp] [Sitia et al., 1987, 1990]). Thus, the presence of a poorly or nonsecretetable protein is necessary but not sufficient to induce the Mott phenotype. The essential difference, we suggest, is in the way such non-secretetable proteins are handled. It is most likely that the cell recognizes malfolded and unassembled polypeptides and quickly degrades them in the ER (Lippincot-Schwartz et al., 1988; Klausner and Sitia, 1990). The problem seems to arise when the ER fails to fully degrade structures that are otherwise unable to proceed through the secretory pathway, as is the case of the mutant IgM which we have studied. Formation of RBs might thus reflect the synthesis of abundant, nonsecretetable molecules which, because of their relative stability, the cell has problems in disposing of.

Why is the mutant IgM not secreted? We have previously proposed that unassembled IgM subunits are retained in a pre-Golgi compartment by disulphide interchange reactions, and that formation of the intersubunit disulphide bonds releases the assembled IgM molecules, which can then proceed through the secretory pathway (Sitia et al., 1990; Alberini et al., 1990). In the case of the mutant, on the contrary, the formation of such bond seems to ultimately lead to the diversion of a stable aggregate into RBs. The critical role of this bond is demonstrated by the fact that substitution of Cys 575 for Ala allows efficient secretion of the double mutant. Reducing agents, albeit less efficiently, also induce
secretion of IgMΔCH1 by both lymphoid and nonlymphoid cells and prevent the formation of RB-like structures in glioma transfectants. The effect of 2ME may be that of preventing formation of polymeric structures while allowing secretion of intermediates.

Our interpretation of the contradictory role of Cys 575 is that the deletion of CH1 leads to noncovalent abnormal polymers. Deletion of the CH1 does not always lead to RBs, or to a total block of secretion (Kohler et al., 1982; Shulman et al., 1982). The specific structure of the VH and/or VL (and perhaps also the light chain isotype) must also play an important role in the formation of abnormal polymers. Such abnormal structures may occur because the hydrophobic phase of the CL domain normally interacts with the CH1 domain. In the absence of this interaction, CLs are likely to interact with each other, as they do in Bence Jones proteins (Milstein, 1965). It is therefore easy to envisage the formation of complexes which depart from the normal H2L2 configuration. This configuration is of the type (VHαVLα) (VHβVLβ) (CLα-CHα1) (CLβ-CHβ1), where α and β are used to symbolize different individual H-L pairs. In the mutant lacking CH1, CLα and CLβ do not need to interact always with each other, but could instead interact with another L chain, which could be either free or already part of a different subunit. An abnormal H-L stoichiometry (see Fig. 5a) would not thus be surprising. This would lead to complex mixtures of aggregates, some recognized as abnormal and degraded. Others however would retain the essential character of normally folded and assembled antibodies, since they bind antigen and interact with antidiotypic antibodies, both requiring VH/VL pairing. The abnormal interactions may be similar to the one previously described in the mutant (IF2), an IgG lacking the CH1 domain (Secher et al., 1977). These abnormal structures, unlike the IgM mutants described here, are secreted, perhaps because they do not further polymerize to give rise to far larger insoluble aggregates. We thus suggest that small noncovalent aggregates form first, which are retained through Cys 575. When S-S bridges between subunits are formed, the resulting product is not a soluble pentamer which can be secreted, but an insoluble aggregate of polymers.

**The Physiological Role of RBs**

If the cell is to survive, it must be capable of handling such insoluble aggregates, which might otherwise block the secretory pathway. Segregating them into RB-like structures is a neat way plasmacells utilize to solve the problem. The fact that nonlymphoid cells are also capable of producing RBs, when faced with a similar problem, suggests that this may be a more general property of secretory cells. The high frequency of RBs in plasmacells may only reflect the high secretory capacity of these cells, coupled to the high frequency of spontaneous mutants within the Ig genes. In addition, lymphoid cells and myelomas are subjected to frequent microscopical examination. However, the problem of disposing of intracellular aggregates in secretory cells is not restricted to the immune system. Particularly relevant here are the recent studies of Tooze and coworkers on pancreatic cells (Tooze et al., 1989, 1990). They have shown that the previously described intracisternal granules (Palade, 1956) are composed of an insoluble aggregate of pancreatic enzymes.
and proenzymes, held together by S-S bonds. Similarly, di-lated ER cisternae, containing under-hydroxylated procolla-gen molecules, have been detected during chondrocyte in vitro differentiation. Addition of ascorbic acid, an essential cofactor in collagen hydroxylation, leads to the progressive vitrodifferentiation. Addition of ascorbic acid, an essential gen molecules, have been detected during chondrocytein and proenzymes, held together by S-S bonds. Similarly, di-mor ortissueculturecells, nor are they terminal cells like tioned that pancreatic cells do not divide at the speed oftu-mor or tissue culture cells, nor are they terminal cells like plasmacells. Thus, formation of RBs (and perhaps intra-cisternal granules) might lead to autophagic vacuoles only when cell division is not sufficient to avoid a constant ac-cumulation incompatible with the cellular architecture.

Since the presence of RBs did not block secretion of L chains or fibronectin, sorting event(s) must occur within the ER which lead to efficient sequestration of the mutant IgM. The phenomenon may present some analogies with sorting events during regulated secretion, occurring however within Golgi cisternae (Kelly, 1985; Hashimoto et al., 1987; Wagen-ner et al., 1987). Whatever the mechanisms implicated in sorting, the RBs appears molecularly distinct from the rest of the ER. Although at the moment we do not know whether this difference is restricted to the segregated contents, or concerns the limiting membranes as well, we feel that RBs should be regarded as subcompartments of the ER. It re-mains to be seen whether RBs correspond to a subregion of the ER present also in normal cells (but perhaps too small to be detected) or they are formed de novo in response to the presence of proteins with altered intracellular traffic.

We are indebted to A. DiFrancesco (Institute of Anatomy, University of Milan, Italy) for his patient and skilful help with the confocal microscope, to C. Alberini, A. Cattaneo, A. Fra, R. Freedman, L. Hendershot, J. F. Kearney, G. Koch, D. Louvard, J. Mason, and M. S. Neuberger for providing cells and reagents, and to A. Cattaneo, A. Fra, L. Inverardi, R. Klausner, J. Meldolesi, M. S. Neuberger, V. Perfetti, and M. Rocco for helpful discussions. C. Valetti and R. Saita thank A. Bargelis, G. Corte and M. E. Cosulich for generously making available laboratory space and equipment. Without the expert secretarial assistance of Mrs. Judy Whybrow, this manuscript would have surely been lost.

This work was in part supported through grants from Fondazione San Romainello, Associazione Italiana per la Ricerca sul Cancro, Consiglio Nazionale Ricerche (Target Projects "Biotechnology and Bioinstrumentation" and "Genetic Engineering," and Special Project "Intracellular Traffic"), and Ministero della Sanità (Special Program on AIDS). C. Valetti is recipi ent of a fellowship from the Fondazione San Romainello.

Received for publication 15 April 1991 and in revised form 12 July 1991.

References

Alalen, A., U. Pira, O. Lassilla, J. Roth, and R. M. Franklin. 1985. Mott cells are plasma cells defective in immunoglobulin secretion. Eur. J. Immunol. 15:253–242.

Alalen, A., U. Pira, A. Colman, and R. M. Franklin. 1987. Mott cells: a model to study immunoglobulin secretion. Eur. J. Immunol. 17:1573–1577.

Alberini, C. M., P. Bet, C. Milstein, and R. Saita. 1990. Secretion of IgM as-sembly intermediates in the presence of reducing agents. Nature (Lond.). 347:485–487.

Bolen, G. D., L. M. Hendershot, and J. F. Kearney. 1986. Post-translational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. J. Cell Biol. 102:1558–1566.

Cattaneo, A., and M. S. Neuberger. 1987. Polymeric immunoglobulin M is secreted by transfectants of non-lymphoid cells in the absence of immunoglobulin J chain. EMBO (Eur. Mol. Biol. Organ.) J. 6:2753–2758.

Cowan, N. J., D. S. Secher, and C. Milstein. 1974. Intracellular immunoglobulin M chain synthesis. J. Mol. Biol. 90:691–701.

Ferrari, M., A. Cadoni, A. T. Franzí, C. Ghiglioni, A. Leprini, A. Zicca, and C. E. Grossi. 1980. Ultrastructure and cytochemistry of human peripheral blood lymphocytes. Similarities between the cells of the third population and To lymphocytes. Eur. J. Immunol. 10:562–570.

Galfré, G., and C. Milstein. 1981. Preparation of monoclonal antibodies: strategies and procedures. Methods Enzymol. 73:3–46.

Hashimoto, S., F. Funagalli, A. Zanini, and J. Meldolesi. 1987. Sorting of three secretory proteins to distinct secretory granules in acidophils of cells from mouse anterior pituitary. J. Cell Biol. 105:1579–1586.

Hendershot, L., D. Bole, G. Köhler, and J. F. Kearney. 1987. Assembly and secretion of heavy chains that do not associate post-translationally with immunoglobulin heavy chain binding protein. J. Cell Biol. 104:761–767.

Kelly, R. B. 1985. Pathways of protein secretion in eukaryotes. Science (Wash. DC). 230:25–32.

Klausner, R. D., and R. Saita. 1990. Protein degradation in the endoplasmic reticulum. Cell. 62:611–614.

Köhler, G., M. J. Potash, H. Lehrach, and M. J. Shulman. 1982. Deletions in immunoglobulin mu chains. EMBO (Eur. Mol. Biol. Organ.) J. 1:555–563.

Laemmlı, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 274:800–585.

Landay, A., L. T. Clement, and C. E. Grossi. 1984. Phenotypically and func-tionally distinct subpopulations of human lymphocytes with T cell markers also exhibit different cytochemical patterns of staining for lysosomal en-zymes. Blood. 63:1067–1071.

Lippincott-Schwartz, J., J. S. Bonifacino, L. C. Yuan, and R. D. Klausner. 1988. Degradation from the endoplasmic reticulum: disposing of newly synthe-sized proteins. Cell. 54:209–220.

Lokhorst, F. M., S. E. Boom, B. J. E. G. Bast, P. J. Peters, T. F. Tedder, J. Gerdes, E. Petersen, and R. E. Balilieux. 1987. Novel type of proliferating lymphoplasmacytoid cell with a characteristic spotted immunofluorescence pattern. J. Clin. Invest. 79:1401–1411.

Maldonado, J. E., J. A. L. Brown, E. D. Boyd, and G. L. Pearse. 1996. Cyto-plasmic and intra-nuclear electron-dense bodies in the myeloma cells. Arch. Pathol. 81:484–500.

Mason, J. O., G. T. Williams, and M. S. Neuberger. 1988. The half-life of immunoglobulin mRNA increases during B-cell differentiation: a possible role for targeting to membrane-bound polysomes. Genes & Dev. 9:1003–1011.

Milstein, C. 1965. Interchain disulphide bridges in Bence-Jones proteins and in γ-globulins B chains. Nature (Lond.). 205:1171–1173.

Mott, F. W., 1905. Observations on the brains of men and animals infected with various forms of trypanosomes. Proc. R. Soc. Lond. 76:235–242.

Neuberger, M. S. 1983. Expression and regulation of immunoglobulin heavy chain transcript into lymphoid cells. EMBO (Eur. Mol. Biol. Organ.) J. 2:1373–1378.

Oi, V., T. S. I. Morrison, L. A. Herzenberg, and P. Berg. 1983. Immunoglobulin gene expression in transformed lymphoid cells. Proc. Natl. Acad. Sci. USA. 80:825–829.

Pacifici, M., and R. V. Iozzo. 1988. Remodeling of the rough endoplasmic reticulum during stimulation of proproliferation by ascorbic acid in cultured chondrocytes. A biochemical and morphological study. J. Biol. Chem. 259:2483–2492.

Palade, G. E. 1956. Intracisternal granules in the exocrine cells of the pancreas. J. Biophys. Biochem. Cytol. 2:417–422.

Pearse, E. G. A. 1949. Nature of the Russell bodies and Kurloff bodies. J. Clin. Pathol. 2:181–88.

Pelham, H. R. B. 1982. A regulatory upstream promoter element in the Dro-sophila hsp 70 heat-shock gene. Cell. 30:517–528.

Potter, H., I. Weir, and P. Leder. 1983. Enhanced-dependent expression of human k immunoglobulin genes introduced into mouse pre-B lymphocytes. Proc. Natl. Acad. Sci. USA. 81:7165–7169.

Reth, M., T. Imanishi-Kari, and K. Rajewsky. 1979. Analysis of the repertoire of anti-4-hydroxy-3 nitrophenyl)acetyl (NP) antibodies in C57 Bl/6 mice by cell fusion. II. Characterization of idiotopes by monoclonal anti-idiotpe antibodies. Eur. J. Immunol. 9:1004–1013.

Russell, W. 1890. Address on a characteristic organism of cancer. Brit. Med. J. 2:1336–1360.

Secher, D. S., C. Milstein, and K. Adetugbo. 1977. Somatic mutants in anti-body diversity. Immunol. Rev. 36:51–72.

Shulman, M. J., C. Heusser, C. Filkin, and G. Köhler. 1982. Mutations affecting the structure and function of immunoglobulin M. Mol. Cell. Biol. 2:1033–1043.

Saita, R. M., S. Neuberger, and C. Milstein. 1987. Regulation of membrane
IgM expression in secretory B cells: translational and post-translational events. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3969–3977.

Sitia, R., M. S. Neuberger, C. M. Alberini, P. Bet, A. M. Fra, C. Valetti, G. T. Williams, and C. Milstein. 1990. Developmental regulation of IgM secretion: the role of the carboxi-terminal cysteine. *Cell.* 60:781–790.

Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26:31–43.

Tacchetti, C., R. Quarto, L. Nitsch, D. J. Hartmann, and R. Cancetta. 1987. In vitro morphogenesis of chick embryo hypertrophic cartilage. *J. Cell Biol.* 105:999–1006.

Tartakoff, A., and P. Vassalli. 1983. Lectin-binding sites as markers of Golgi subcompartment: proximal to distal maturation of oligosaccharides. *J. Cell Biol.* 97:1243–1248.

Tooze, J., H. F. Kern, S. D. Fuller, and K. E. Howell. 1989. Condensation-sorting events in the rough endoplasmic reticulum of exocrine pancreatic cells. *J. Cell Biol.* 109:35–50.

Vertel, B. M., A. Velasco, S. LaFrance, L. Walters, and K. Kaczman-Daniel. 1989. Precursor of chondroitin sulphate proteoglycan are segregated within a subcompartment of the chondrocyte endoplasmic reticulum. *J. Cell Biol.* 109:1827–1836.

Wagner, D. D., S. Saffaripour, R. Bonfanti, J. E. Sadler, E. M. Cranker, B. Chapman, and T. N. Mayadas. 1991. Induction of specific storage organelles by von Willebrand factor propolypeptide. *Cell.* 64:403–413.

Weiss, S., P. D. Burrows, J. Meyer, and M. R. Wabl. 1984. A Mott cell hybridoma. *Eur. J. Immunol.* 14:744–748.

Zicca, A., A. Cadoni, A. Leprini, R. Millo, P. M. Lydyard, and C. E. Grossi. 1982. Immunofluorescent and ultrastructural analysis of plasma cell degeneration in the chicken harder’s gland. *Dev. Comp. Immunol.* 6:131–139.

Ziegler, A., and H. Hengartner. 1977. Sodium dodecyl-sulfate electrophoresis of high molecular weight proteins in N,N’ diallyl tartar diamide—cross—linked polyacrylamide slab gels. *Eur. J. Immunol.* 7:690.