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Macromolecular aggregation of F1 and P1 fractions of RU 41740 an immunomodulating compound isolated from *Klebsiella pneumoniae*, as revealed by size exclusion high-performance liquid chromatography and light scattering detection

Karine Nimier *, Daniel Robic, Richard Bourbouze

Laboratoire de Biochimie, Faculté des Sciences Pharmaceutiques et Biologiques, Université René Descartes, 4 avenue de l'Observatoire, 75006 Paris, France

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Abstract

RU 41740 isolated from *Klebsiella pneumoniae* possesses high immunomodulating activity. This immunomodulating agent has been described as composed of two glycoprotein F1 and P1 fractions. Some characteristics of native RU 41740 and F1 and P1 fractions were investigated using size exclusion high-performance liquid chromatography and light scattering detection. Particle size distribution and apparent molecular mass data suggest that RU 41740 is a macromolecular aggregate of numerous F1 and P1 subunits. Findings suggest an effect of orally administered RU 41740 on the function of Peyer's patch cells.

Key words: RU 41740; Immunomodulating compound; Macromolecular size; Oral absorption; Light scattering

1. Introduction

RU 41740 (trade name Biostim) is a glycoprotein complex isolated from *Klebsiella pneumoniae* strain O_1K_2 (Laboratoires Cassenne, Roussel-UCLAF, France). Treatment of this compound with cetyltrimethylammonium chloride leads to the production of two subunits F1 and P1. The soluble glycoprotein F1 fraction of 350 kDa contains only neutral sugars, while the precipitating glycoprotein P1 fraction of 95 kDa contains glucuronic acid units (Griscelli et al., 1982; Kol et al., 1987).

RU 41740 is an immunomodulating compound with a broad spectrum of activity and is clinically used for the treatment of chronic bronchitis and recurrent pulmonary infections (Viallat et al., 1983; Anthoine et al., 1985; Bonde et al., 1986). It is now well known that RU 41740 has a marked ability to increase phagocytosis and cell-mediated and humoral immune responses of animals and humans (Nielsen and Bonde, 1986; Viland and Biomgren, 1987). Treatments with RU 41740 are administered orally, therefore, its activity, mediated by a local effect on the gut-associated lymphoid tissue, is an important question.

* Corresponding author.

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The literature now contains substantial evidence that the uptake of microparticulate material and macromolecules takes place by endocytic mechanisms, at the M-cells of Peyer's patches (Robert and Owen, 1977; Pappo et al., 1991). This observation is of particular interest considering that Peyer's patches are secondary lymphoid organs which are believed to deliver antigenic information from the luminal content to the underlying lymphocytes and initiating immune responses (Butcher et al., 1982). Recently, using oral absorption of polystyrene nanospheres in the rat, it has been demonstrated that their translocation and subsequent concentration in the lymphatic tissues of the reticuloendothelial system are clearly functions of size; 1 μm polystyrene microspheres were the largest which were taken up by Peyer's patches and subsequently found in the lymph nodes and other internal organes (liver and spleen), while smaller (50 nm size diameter) microspheres can be taken up not only by Peyer's patches but also by enterocytes in the villous part of the gastrointestinal tract (Jani et al., 1992a,b).

Consequently, this study focuses primarily on the molecular size of RU 41740 in its pharmaceutical form under physiological conditions. In the present report some characteristics of the glycoprotein complex RU 41740 have been investigated using size exclusion high-performance liquid chromatography and dynamic light scattering for detection, which are suitable techniques in estimating the particle size of macromolecules and their degree of aggregation. The results suggest that the immunomodulating agent RU 41740 is a macromolecular aggregate of numerous F1 and P1 subunits.

2. Materials and methods

2.1. Specimens

Specimens used in this study, RU 41740 and the corresponding F1 and P1 fractions were supplied by Laboratoires Cassenne, Osny, France. F1 and P1 fractions used for experiments came from the same batch of RU 41740.

2.2. High-performance liquid chromatography

The Pharmacia Fast Protein Liquid Chromatography (FPLC) system was used. Prepacked Superose^{TM}12 and 6 columns were equilibrated and eluted with 0.05 M sodium phosphate buffer pH 7, 0.1 M NaCl. Degassed eluant was pumped at a rate of 0.25 and 0.5 ml/min, respectively. Specimen solutions were prepared by dissolving 10 mg/ml of lyophilized powder in 0.05 M sodium phosphate buffer pH 7, 0.1 M NaCl and stirred overnight at room temperature. Samples of 0.2 ml were applied to the columns and 0.5 ml fractions were collected. Elution profiles were detected at 280 nm. V_0 (void volume of the column) is estimated using thyroglobulin (669 kDa) for Superose^{TM}12 and a rat liver mitochondrial fraction for Superose^{TM}6. V_t, the total volume of the column, is estimated using vitamin B12 (1.3 kDa). MPB buffer, a non-aggregating buffer solution, was composed of 18 mM Hepes, 7 mM imidazole, 1 mM EDTA (sodium salt), 3 mM sodium azide, 200 mM sodium acetate and 0.5 mM non-ionic surfactant octaethylene glycol mono-*N*-dodecyl ether; Sigma). Results are shown from single experiment and were representative of several separate experiments.

2.3. Light scattering

The Oros Instruments Model 801 Molecular Size Detector coupled to the liquid chromatography system was used. This equipment is based on the quasi-elastic principle, also being referred to as dynamic light scattering. Injections were made in phosphate buffer system at concentrations ranging from 5 to 10 mg/ml. The specimens reconstituted in buffer (as above) from freeze-dried material or eluate from liquid chromatography system are held in a cell (7 μl) and illuminated by a focused laser beam every 10 s. The total volume of sample required is at least 40 μl to ensure that the cell and liquid line are completely flushed with fresh solution. Scattered photons are collected and transmitted to a photomultiplier tube detector. The time scale of the scattering light intensity fluctuation is analysed by autocorrelation (Nicoli et al., 1992). The detector

is calibrated with a 5 mg/ml BSA in 0.1 M PBS solution, filtered 0.1 μm (R_H, 3.7 nm; molecular mass, 68 kDa).

3. Results

The chromatographic patterns of RU 41740 on Superose^{TM}12 is presented in Fig. 1. Chromatography on Superose^{TM}12 resolves RU 41740, according to the elution profile at 280 nm, into two peaks corresponding to a major, apparently heterogeneous, high molecular mass fraction and a low molecular mass fraction: peak I, usually in the range V_e/V_o 0.8–1.6; peak II, V_e/V_o 2.2–2.4. It is noteworthy that further chromatography of peak I under the same conditions shows an elution profile in two fractions similar to that obtained with native RU 41740. Fractionation on Superose^{TM}12 of F1 and P1 fractions (from RU 41740) under the same conditions resolves F1 as a single peak (Fig. 2A) in the void volume range of the column (i.e., > 300 kDa). By contrast, P1 shows a complex profile suggesting aggregation of this fraction (Fig. 2B).

The formation of macromolecular aggregates of F1 and P1 fractions was further examined using chromatography on Superose^{TM}6 in different buffer systems, phosphate buffer and MPB. We chose MPB buffer, which causes no aggregation of protein upon dissolution (Magiera and Krull, 1992). Attempts to fractionate peak I from Superose^{TM}12 RU 41740 chromatography were unsuccessful with the two system buffers. Thus, chromatography on Superose^{TM}6 resolves RU 41740 into two peaks corresponding to the high molecular mass fraction in the void volume of the column (V_e/V_o 0.9–1.3, i.e., $> 5 \times 10^3$ kDa) and low molecular mass fraction (V_e/V_o 2.6–3.1) (Fig. 3). On the basis of these chromatographic data, a macromolecular aggregate of F1 and P1 fractions

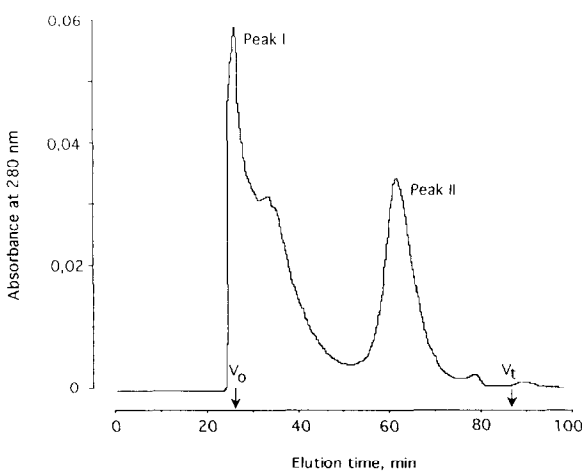

Fig. 1. Typical chromatographic pattern (A_{280} nm) of RU 41740 (2 mg) on Superose^{TM}12. Chromatography was carried out as described in section 2. V_o, void volume of the column estimated using thyroglobulin (669 kDa); V_t, total volume of the column estimated using vitamin B12 (1.3 kDa).

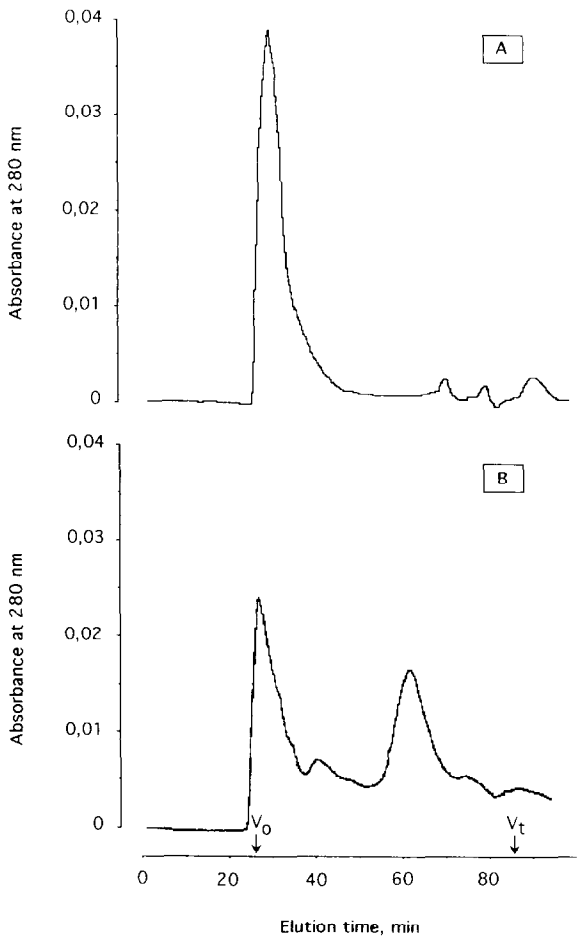

Fig. 2. Typical chromatographic patterns of (A) F1 fraction (2 mg) and (B) P1 fraction (1 mg) from RU 41740 on Superose^{TM}12.

from RU 41740 is probably present in lyophilized powder before dilution by chromatographic elution. The degree of aggregation remains important in spite of partial dissociation due to chromatography on Superose™12 and 6.

Several recent studies have reported expression of membrane alkaline phosphatase activity on activated B cells, associated with cell progression to differentiation and consequent IgM synthesis (Burg and Feldbush, 1989; Feldbush and Lafrenz, 1991). We have previously demonstrated that incubating mouse spleen cells with RU 41740 results in cell proliferation and expression of high levels of alkaline phosphatase activity. This effect was dose dependent and hence alkaline phosphatase appears to be a marker of B lymphocyte activation by RU 41740 (Nimier et al., unpublished data). It is noteworthy that the P1 fraction used alone was inactive compared to the response given by RU 41740 or the F1 fraction. The eluates corresponding to peaks I and II from chromatography of RU 41740 on Superose™12 (Fig. 1) were desalted by repeated dialysis using distilled water and lyophilized. We have observed expression of alkaline phosphatase activity by murine B lymphocytes exposed to the two peaks but peak I alone was as active as RU 41740. These results strengthen the chromatographic data suggesting aggregates of F1 and P1 for both the two peaks obtained by size exclusion chromatography. Different degrees of aggregation or modifications of macromolecular organization during the fractionation procedure can explain the fact that peak II activity is weaker than that of the entire extract.

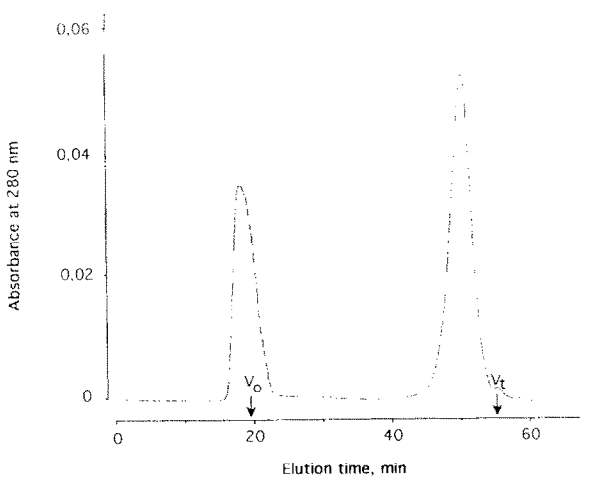

Fig. 3. Typical chromatographic pattern of RU 41740 (2 mg) on Superose™6. Elution with MPB or phosphate buffer.

Table 1
Particular size and apparent molecular mass from RU 41740 and F1 and P1 fractions as revealed by light scattering

Specimens	Hydrodynamic radius (nm)	Apparent molecular mass (MDa)
RU 41740		
FPLC	26–278	8 – 2400
Fraction F1		
direct injection	48	35
FPLC	9–116	0.67 – 240
Fraction P1		
direct injection	160–200	600 – 1200
FPLC	12–254	1 – 1900

Hydrodynamic radii from the molecular mass detector were measured by direct injection of specimen solutions or of peaks eluted in the void volume from Superose™6 chromatography (FPLC). The detector was calibrated using BSA (hydrodynamic radius, 3.7 nm; molecular mass, 68 kDa). Further experimental details are described in section 2.

The extent of aggregation of RU 41740 components was examined by size exclusion high-performance liquid chromatography with low-angle light scattering detection. The hydrodynamic radius of the pure F1 and P1 solutions was measured by direct injection into the molecular size detector; a specimen of RU 41740 was not used due to sample viscosity. Light scattering of the high molecular mass fraction eluted in the void volume of Superose™6 chromatography was measured for RU 41740, F1 and P1; the low molecular mass fraction from Superose™6 chromatography was not used because of sample limitations for this study. The particle size distribution (nm) and molecular mass-range (MDa) results are listed in Table 1 for RU 41740, and F1 and P1 fractions. From these data, the hypothesis of a three-component reacting system can be proposed; aggregation of P1 itself and interaction between aggregates of F1 and P1 fractions. It was found that the molecular size of the complexes, with large-scale distribution, varied on elution

from Superose™6. Dilution by chromatography decreases the interaction forces between the components and significant dissociation of the complexes was observed. Therefore, RU 41740 appeared as a macromolecular aggregate of F1 and P1 fractions in the lyophilized powder before dilution.

4. Discussion

Taken together, the chromatographic and light scattering data led to the conclusion that the immunomodulating agent RU 41740 may be a macromolecular aggregate of the two major fractions F1 and P1.

At this stage, a few remarks concerning the association process between F1 and P1 may be suitable. One might expect that both kinds of protein-polysaccharide molecules should contain many reactive sites and thus form large polymeric aggregates. So, previous studies have shown that certain glycosaminoglycans bearing glucuronic acid units like P1 fraction of RU 41740 bind electrostatically to soluble collagen under physiological conditions. In the interaction between collagen and dermatan sulfate, the polysaccharide chain could bind several collagen molecules which in turn can bind several dermatan sulfate molecules (Öbrink and Sundelöf, 1973). Recently, using chromatographic procedures and light scattering, the formation of aggregates of alkaline phosphatase (Magiera and Krull, 1992) and the interaction between acid glycosaminoglycans and proteins in normal human plasma (Calatroni et al., 1992) have also been demonstrated.

As revealed by chromatographic results from other batches, the degree of aggregation in RU 41740 remained constant, probably being present in lyophilized powders before partial dissociation by chromatographic dilution or intestinal transport (Heyman et al., 1987). Particle size determines selective localisation in Peyer's patches. It has been assumed that the M-cells of Peyer's patches are involved in the absorption of SSG, a β-glycan immuno-adjuvant, since SSG has a characteristic high molecular mass ($> 5 \times 10^3$ kDa) compared with other β-glycans which are ineffective via the oral route (Hashimoto et al., 1991). On the other hand, the average molecular mass of mytilan, a bioglycan immunomodulator isolated from mussel (*Crenomytilus grayanus*), was estimated to be 3×10^3 kDa (Ovodova et al., 1992). Our present findings suggest a pathway for specifically targeting RU 41740 to M-cells for delivery to mucosal lymphoid tissue.

5. Acknowledgments

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6. References

Anthoine, D., Biaive, B., Cabanieu, G. and Chretien, J., Etude en double aveugle du Biostim® dans la prévention des surinfections des patients atteinds de bronchopathie chronique. *Rev. Pneumol. Clin.*, 41 (1985) 213–217.

Bonde, J., Dahl, R., Edelstein, R., Kok-Jensen, A., Lazer, L., Punakivi, L., Seppala, A., Soes-Petersen, U. and Viskum, M., The effect of RU 41740, an immunomodulating compound in the prevention of acute exacerbations in patients with chronic bronchitis. *Eur. J. Respir. Dis.*, 69 (1986) 235–241.

Burg, D.L. and Feldbush, T.L., Late events in B cell activation: expression of membrane alkaline phosphatase activity. *J. Immunol.*, 142 (1989) 381–387.

Butcher, E.C., Rouse, R.V., Coffman, R.L., Nottenburg, C.N., Hardy, R.R. and Weissman, I.L., Surface phenotype of Peyer's patch germinal centers in B cell differentiation. *J. Immunol.*, 129 (1982) 2698–2706.

Calatroni, A., Vinci, R. and Ferlazzo, M., Characteristics of interactions between acid glycosaminoglycans and proteins in normal human plasma as revealed by the behavior of the protein-polysaccharide complex in ultrafiltration and chromatographic procedures. *Clin. Chim. Acta*, 206 (1992) 167–180.

Feldbush, T.L. and Lafrenz, D., Alkaline phosphatase on activated B cells. Characterization of the expression of alkaline phosphatase on activated B cells. *J. Immunol.*, 147 (1991) 3690–3695.

Griscelli, C., Grospierre, B., Montreuil, J., Fournet, B., Bruvier, C., Lang, J.M., Marchiani, C., Zalisz, R. and Edelstein, R., Immunomodulation by glycoprotein fractions isolated from *Klebsiella pneumoniae*. In Yamamura, Y. and Kotani, S. (Eds), *Immunomodulation by Microbial Products and Related Compounds*, Excerpta Medica, Amsterdam, 1982, pp. 261–265.

Hashimoto, K., Suzuki, I. and Yadomae, T., Oral administration of SSG, a β-glucan obtained from *Sclerotinia sclerotorium*, affects the function of Peyer's patch cells. *Int. J. Immunopharmacol.*, 13 (1991) 437–442.

Heyman, M., Bonfils, A., Fortier, M., Crain-Denoyelle, A.M., Smets, P. and Desjeux, J.F., Intestinal absorption of RU 41740, an immunomodulating compound extracted from *Klebsiella pneumoniae*, across duodenal epithelium and Peyer's patches of the rabbit. *Int. J. Pharm.*, 37 (1987) 33–39.

Jani, P.U., Florence, A.T. and McCarthy, D.E., Further histological evidence of the gastrointestinal absorption of polystyrene nanospheres in the rat. *Int. J. Pharm.*, 84 (1992a) 245–252.

Jani, P.U., McCarthy, D.E. and Florence, A.T., Nanosphere and microsphere uptake via Peyer's patches: observation of the rate of uptake in the rat after a single oral dose. *Int. J. Pharm.*, 86 (1992b) 239–246.

Kol, O., Montreuil, J., Fournet, B., Zalisz, R. and Smets, P., Purification of the lipopolysaccharide fraction from *Klebsiella pneumoniae* O1K2 by high performance liquid chromatography. *J. Chromatogr.*, 396 (1987) 281–286.

Magiera, D.J. and Krull, I.S., Determination of alkaline phosphatase aggregation by size exclusion high-performance liquid chromatography with low-angle laser light scattering detection. *J. Chromatogr.*, 606 (1992) 264–271.

Nicoli, D.F., McKenzie, D.C. and Wu, J.S., Dynamic light scattering size analysis of macromolecules. *Int. Lab.*, 22 (1992) 32–37.

Nielsen, H. and Bonde, J., Immunostimulation of blood monocyte function by RU 41740 (Biostim®) in patients with chronic bronchitis. *Int. J. Immunopharmacol.*, 8 (1986) 589–592.

Öbrink, B. and Sundelöf, L.O., Light scattering in the study of associating macromolecules. *Eur. J. Biochem.*, 37 (1973) 226–232.

Ovodova, R.G., Glazkova, V.E., Mikheyskaya, L.V., Molchanova, V.I., Isakov, V.V., Ovodov, Y.S. and Molina, L.F., The structure of mytilan, a bioglycan-immunomodulator isolated from the mussel *Crenomytilus grayanus*. *Carbohydr. Res.*, 223 (1992) 221–226.

Pappo, J., Ermak, T.H. and Steger, H.J., Monoclonal antibody-directed targeting of fluorescent polystyrene microspheres to Peyer's patch M cells. *Immunology*, 73 (1991) 277–280.

Robert, L. and Owen, M.D., Sequential uptake of horseradish peroxydase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. *Gastroenterology*, 72 (1977) 440–451.

Viallat, J.R., Costantini, D., Boutin, C. and Farnisse, P., Etude en double aveugle d'un immunomodulateur d'origine bactérienne (Biostim®) dans la prévention des épisodes infectieux chez les bronchitiques chroniques. *Poumon Coeur*, 39 (1983) 53–57.

Viland, H. and Biomgren, H., Augmentation of spontaneous cytotoxicity of human lymphocytes by RU 41740 is due to monocyte-derived factors distinct from interleukin 2, interferon alpha and gamma. *Anticancer Res.*, 7 (1987) 23–28.