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Immunogenic and tolerogenic properties of monomethoxypoly(ethylene glycol) conjugated proteins

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Abstract

An immunogenic and tolerogenic characterisation of monomethoxypoly(ethylene glycol) conjugated proteins was carried out using, as immunogen models, an anti-malaria chimera monoclonal antibody (PfChMab) and a macrophage colony stimulating factor (M-CSF). Two conjugates of PfChMab were prepared by polymer derivatisation of 19 and 33% protein amino groups and one conjugate of M-CSF was obtained by modification of 24% amino groups. In mice M-CSF was found to elicit rapidly high IgG and IgM levels whereas the monomethoxypoly(ethylene glycol) derivatised M-CSF stimulated a significantly lower immunoresponse. Native PfChMab was found to induce a delayed immunoresponse with high IgM levels but low production of IgG. Furthermore, similar immunogenic profiles were obtained with the native and modified protein forms. The pre-administration of polymer conjugated M-CSF to mice subsequently treated with the native protein was found to suppress up to 75% of anti-native M-CSF IgG, while IgM production was not affected. On the other hand the pre-administration of monomethoxypoly(ethylene glycol) derivatised PfChMab was found to reduce significantly the generation of anti-native PfChMab IgM. Such suppression depended on the degree of modification: the conjugate with the higher number of polymer chains was more effective in suppressing the immunoresponse.

Keywords: Tolerance; Immunogenicity; Polymer conjugate

1. Introduction

The covalent conjugation of soluble and compatible polymers to protein and peptide surface has been largely and successfully used to enhance the therapeutic potential of monoclonal antibodies, cytokines, hormones, enzymes, immunotoxins and other xenogenic biomodulators. Indeed, protein–polymer conjugates often display, with respect to the native counterparts, prolonged permanence in blood, reduced immunogenicity and increased stability and solubility [1–5].

Monomethoxypoly(ethylene glycol) (PEG) is the polymer of choice for such purpose because it possesses suitable physico–chemical and biological properties and the main pre-requisites for pharmaceutical application, namely absence of local and systemic toxicity and low immunogenicity. For these reasons several protein–PEG conjugates have been developed and some of them are in advanced clinical study [6–9].

However, despite the advantages of this technique, polymer attachment can in some cases induce loss or reduction in protein biological activity that seriously prevents the exploitation of these drugs. The covalent polymer binding to the protein surface can in fact provoke conformational changes in the protein molecular structure as well as the polymeric cloud around the protein can prevent the interaction of the construct with the receptor or substrate [10–12].

Recently, polymer conjugation has been also proposed for development of tolerogenic molecules. Several studies demonstrate in fact that the pre-administration of polymer–protein conjugates can abrogate the primary antibody response towards further treatment with the native counterpart. Therefore, it has been suggested that polymer conjugation may switch allergenic molecules into tolerogenic compounds [13,14].

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This fascinating hypothesis has been actively investigated using various immunogens, animal models, immunisation protocols and polymers for the immunogen conjugation [13–16]. However, although many evidences point out a selective downregulation of the immunoresponse to the antigen, the results reported in literature are often controversial. Furthermore the mechanism underlying the immunosuppression has not been fully explained probably because of the complexity of the immunosystem, the high individual variability and the many parameters that dictate the final immunoresponse [17–22]. In particular, relatively low attention has been paid so far to evaluate the requisites that the immunogen and its polymer conjugate must possess to display tolerogenic properties.

This study deals with the possibility to exploit low immunogenic and tolerogenic derivatives of immunogenic proteins of medical interest. In particular this research is aimed at elucidating the possible relationship between the immunological properties of the native molecule and the immunogenic and tolerogenic character of its conjugate. In the present investigation two therapeutically useful proteins were used as immunogen protein models: a macrophage colony stimulating factor (M-CSF) and an anti-malaria monoclonal antibody (PfChMab). These proteins were chosen because they were found to possess different immunogenic potentials; in mice M-CSF stimulates a rapid and intense immunoresponse, whereas a lower and delayed antibody production is obtained by PfChMab immunisation. The immunogenic and tolerogenic study was carried out in mice with M-CSF and PfChMab derivatives obtained by covalent conjugation of monomethoxypoly(ethylene glycol) 5000 Da mol. wt. (PEG): two PfChMab–PEG conjugates bearing a different number of polymer chains and one M-CSF–PEG derivative.

2. Materials and methods

Monomethoxypoly(ethylene glycol) 5000 was obtained from Fluka (Buchs, Switzerland). Complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) and all the other reagents were furnished from Sigma (St. Louis, MO). The FPLC system and the analytical Superose 6 column were furnished from Pharmacia Biotech (Uppsala, Sweden). The ELISA microplates and the microplate reader system were from Dynatech Laboratories (Chantilly, VA). Peroxidase conjugated goat anti-mouse antibodies (anti-IgG, anti-IgM and anti-[IgG + IgM]) were purchased from Boerhinger (Steinheim, Germany). Three-week-old female Balb/c mice fed 'ad libitum' used for 'in vivo' experiments were obtained by Charles River. Macrophage colony stimulating factor (M-CSF) and chimera anti-malaria monoclonal antibodies (PfchMab) were a kind gift of SmithKline and Beecham Laboratories (King of Prussia, PA).

2.1. Monomethoxypoly(ethylene glycol)–protein preparation

Monomethoxypoly(ethylene glycol) (PEG) 5000 Da was end carboxylated by introduction of a norleucine residue (Nle) and the COOH functional group was further activated as succinimidyl ester according to the method reported in literature [23].

2.1.1. M-CSF–PEG preparation

Activated polymer (8.5 mg) was added, under vigorous stirring, to 3 mg of M-CSF dissolved in 2 ml of 0.2 M borate buffer, pH 8.0, in order to reach a polymer/available protein amino group molar ratio of 1:1. The reaction solution was gently stirred for 2 h at room temperature (r.t.) and the polymer–protein conjugate was purified by gel permeation chromatography using an analytical Superose 6 column eluted with 0. 01 M phosphate buffer, 0.15 M NaCl, (PBS), pH 7.2. The elution volume was analysed by O.D. at 280 nm for protein detection and by iodine test for the polymer determination [23]. The polymer–protein peak was collected, concentrated by Centriprep 10 (Amicon, Danvers, NA) and the number of polymer chains bound on the protein surface was evaluated by amino acid analysis [24] and expressed as the percent of the derivatised protein NH_2/total available NH_2 (degree of modification).

2.1.2. PfChMab–PEG preparation

Proper amounts of activated PEG (9 or 13.5 mg) were added under vigorous stirring to 2 ml of 0.2 M borate buffer, pH 8.0, solutions containing 3 mg of PfChMab in order to reach polymer–protein amino group molar ratios of 1:1 or 1.5:1. The PfChMab–PEG derivatives were purified and analysed as reported above for M-CSF–PEG preparation.

2.2. Antibody level determinations

The ELISA 96-well microplates were incubated overnight at 4°C with 100 μl/well of coating solution (0.1 M $NaHCO_3$, pH 9.5) containing 100 μg/ml of native protein (M-CSF or PfChMab). The plates were washed three times with 200 μl/well of PBS/0.05% Tween v/v, pH 7.2 (PBS-T) and further incubated for 2 h at r.t. with 200 μl of ELISA blocking reagent (Pierce, Rockford, IL). After washing, the wells were incubated for 2 h at r.t. with 100 μl of serum samples serially diluted in PBS-T, washed and incubated as above with 100 μl of peroxidase conjugated goat anti-mouse antibodies (anti-IgG or anti-IgM or anti-[IgG + IgM])

properly diluted in PBS-T. After washing 100 μl of 0.1 M citrate buffer, pH 4.5, containing 1 mg/ml of OPD and 0.4 μl/ml of H_2O_2 35% were added for 10 min and the enzymatic reaction was stopped by adding 100 μl 0.1 N NaF. The wells were finally read by O.D. at 450 nm.

2.3. Immunogenic evaluations

2.3.1. M-CSF

On day 0, five Balb/c mice were immunised with 25 μg of native M-CSF dissolved in 200 μl of PBS, pH 7.2/CFA (50/50 v/v) (Group 1) and five mice were immunised with 40 μg of M-CSF–PEG dissolved in 200 μl of the same buffer/CFA mixture (Group 2). On days 7 and 14, the animals of Group 1 were boosted with 12.5 μg of M-CSF dissolved in 100 μl of PBS, pH 7.2/IFA (50/50, v/v) and the animals of Group 2 were boosted with 20 μg of M-CSF–PEG in 200 μl of the same buffer/IFA mixture. All the animals were bled on days 7, 14 and 21 and serum was analysed for determination of anti-native MCSF [IgG + IgM], IgG and IgM levels by ELISA as reported above.

2.3.2. PfChMab

On day 0, five Balb/c mice were immunised with 50 μg of native PfChMab dissolved in 200 μl of PBS, pH 7.2/CFA (50/50 v/v) (Group 1), five mice were immunised with 81 μg of PfChMab–PEG19 (Group 2) and five animals with 105 μg of PfChMab–PEG33 (Group 3) in 200 μl of the same buffer/CFA mixture. The animals were boosted on days 7, 14, 21 and 28 with 25 μg/mouse of native PfChMab (Group 1), 40 μg/mouse of mPEG19–PfChMab (Group 2), and 52 μg/mouse in 200 μl of PBS, pH 7.2/IFA (50:50, v/v). The mice were bled on days 7, 14, 21, 28 and 35 and both anti-native PfChMab IgG and anti-native PfChMab IgM levels in serum were estimated by ELISA as reported above.

All immunisations were performed by injecting in mice half of the volume of the immunising solution intraperitoneously and the other half subcutaneously. Blood was taken by retrobulbar bleeding after anaesthesia and serum was obtained by blood centrifugation at 3500 rpm for 3 min.

2.4. Tolerogenic evaluations

2.4.1. M-CSF (A)

Fifteen Balb/c mice were divided into three groups of five animals each (Control Group A, Control Group B, and the Test Group). The animals were pre-treated with: 200 μl of PBS, pH 7.2 (Control Group A), 100 μg of not activated PEG in 200 μl of PBS, pH 7.2 (Control Group B) and 200 μg of M-CSF–PEG in 200 μl of PBS, pH 7.2 (Test Group). After 7 days, day 0, the mice were immunised with 25 μg of M-CSF in 100 μl of PBS, pH 7.2 and 100 μl of CFA and boosted on days 7 and 14 with 12.5 μg of M-CSF in 100 μl of PBS, pH 7.2 and 100 μl of IFA. The animals were bled 7 days after each immunisation and anti-native M-CSF [IgG + IgM] were evaluated in serum plasma by ELISA.

2.4.2. M-CSF (B)

Twenty Balb/c mice were divided into four groups of five animals each (Groups 1–4). The animals of Groups 1 and 3 were pre-treated with 100 μg of PEG in 200 μl of PBS, pH 7.2 and the animals of Groups 2 and 4 were pre-treated with 200 μg of M-CSF–PEG in 200 μl of PBS, pH 7.2. After 7 days, day 0, the animals of Groups 1 and 2 were immunised with 25 μg of native M-CSF in 200 μl of PBS, pH 7.2, and boosted on days 7, 14, 21, 35, 49, 63 and 77 with 12.5 μg of native M-CSF in 200 μl of the same buffer. The animals of Groups 3 and 4 were immunised on day 0 with 25 μg/mouse of M-CSF in 200 μl of PBS, pH 7.2/CFA (50/50) and boosted on days 7, 14, 21, 35, 49, 63 and 77 with 12.5 μg of MCSF in 200 μl of PBS, pH 7.2/IFA (50/50). The animals were bled at scheduled days and anti-native M-CSF IgM and anti-native M-CSF IgG were separately evaluated in serum after proper dilution by ELISA.

2.4.3. PfChMab

Fifteen Balb/c mice were divided into three groups of five animals each (Groups 1–3). The mice of Group 1 were pre-treated with 100 μg of PEG in 200 μl of PBS, pH 7.2, the mice of Group 2 with 200 μg/mouse of PfChMab–PEG19 in 200 μl of PBS, pH 7.2 and the animals of Group 3 with 200 μg/mouse of PfChMab–PEG33 in 200 μl of PBS, pH 7.2. All the animals were immunised on days 0, 7, 14, 21, 28, 42, 49, and 63 with 25 μg of native PfChMab in 200 μl of PBS, pH 7.2, and bled at scheduled days. Anti-native PfChMab IgM and anti-native PfChMab IgG were separately evaluated in the serum samples by ELISA.

All the volumes administered to the animals were injected half intraperitoneously and half subcutaneously.

2.5. Mathematical analysis

In immunogenicity and tolerogenicity evaluations, standard deviation ($\pm$ SD) has been calculated on the basis of the single animal data.

The tolerogenic effect of the polymer conjugates has been expressed, according to literature [21,22], as percent of inhibition in antibody generation that was calculated as follows:

$$(1 - A/B) \times 100$$

where A = geometric means of the dilution giving 0.4 O.D. at 450 nm in the test and B = geometric means of the dilution giving 0.4 O.D. at 450 nm in the control. The significativity of the tolerogenic data was estimated on the basis of Student's *t*-test.

3. Results

For this study, three different monomethoxypoly(ethylene glycol)–protein conjugates were prepared: MCSF–PEG, PfChMab–PEG19 and PfChMab–PEG33. Table 1 reports the conjugation reaction conditions (polymer/protein amino group molar ratios) and the main properties of the conjugates: percent of derivatised protein amino groups (degree of modification) and molecular weight of the various protein forms. The two PfChMab–polymer conjugates, PfChMab–PEG19 and PfChMab–PEG33, were obtained by using proper activated PEG/protein NH_2 group molar ratios. The amino acid analysis of the derivatives demonstrated that an average of 19 and 33 PEG chains per protein molecule were respectively bound in the PfChMab–PEG19 and PfChMab–PEG33 derivatives. The M-CSF–PEG was obtained by modification of 24% of protein amino groups that corresponds to eight polymer chains per M-CSF molecule.

3.1. Immunological properties of the monomethoxypoly(ethylene glycol) conjugates

Fig. 1 describes the immunogenic profiles obtained by mouse treatment with native M-CSF and M-CSF–PEG. Freund's adjuvant was used in the immunisation protocol and the anti-native M-CSF [IgG + IgM] levels were estimated 1 week after each immunisation. The figure indicates that the PEG derivatised form elicits a lower anti-M-CSF immunoresponse as compared to the native counterpart. After the third immunisation, the difference in anti-M-CSF antibody levels between the two protein forms was found to be maximal. At this

Table 1
Reaction conjugation conditions, degree of protein modification and molecular weight of M-CSF–PEG, PfChMab–PEG19 and PfChMab–PEG33

	Activated polymer/available protein NH_2 group (molar ratio)	Degree of modification (% of modified amino groups)	Molecular weight (Da)
M-CSF		0	60 000
M-CSF–PEG	1:1	24	100 000
PfChMab		0	150 000
PfChMab–PEG19	1:1	19	245 000
PfChMab–PEG33	1.5:1	33	315 000

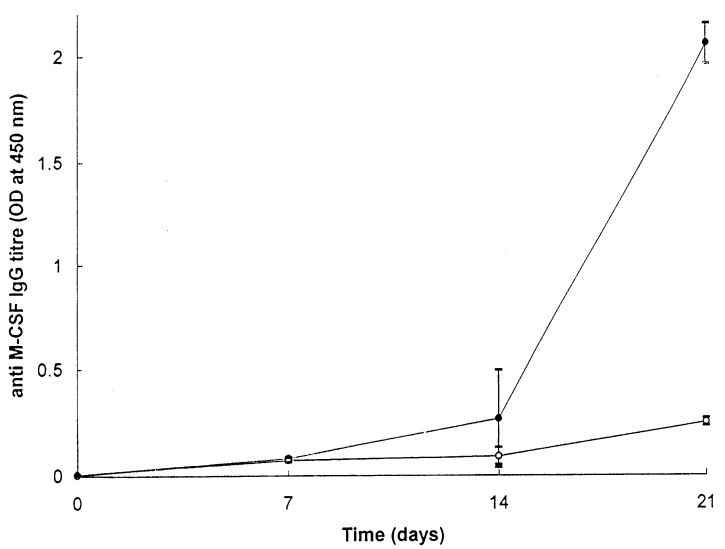

Fig. 1. Immunogenic time course of native M-CSF (●) and M-CSF–PEG (○). The [IgG + IgM] immunoresponse was evaluated by ELISA at days 0, 7, 14 and 21 from the 1st immunisation and has been expressed as O.D. at 450 nm of 1:160 serum dilutions.

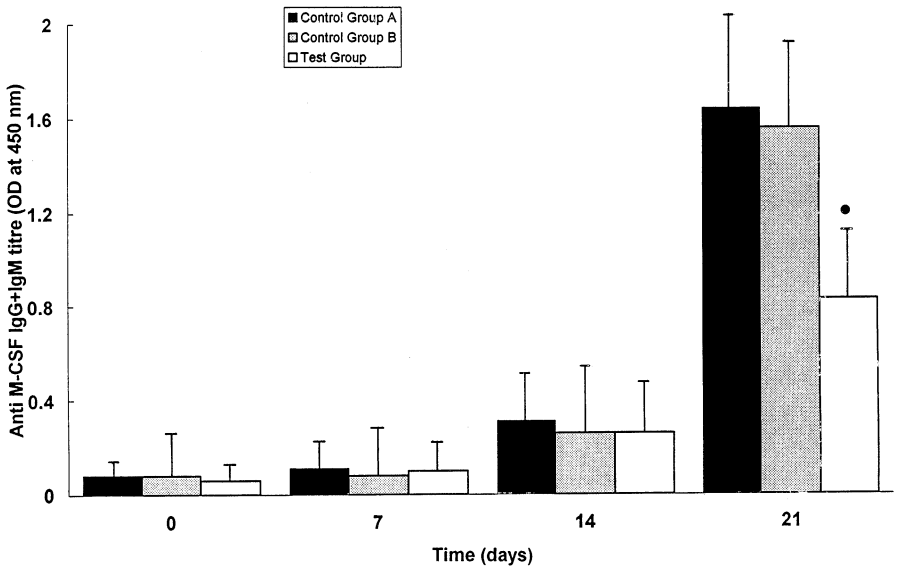

Fig. 2. Tolerogenic effect of M-CSF–PEG: suppression of anti-native M-CSF [IgG + IgM] production. Animals were pre-treated with PBS only (Control Group A), non activated PEG (Control Group B) and M-CSF–PEG (Test Group). The antibody levels were evaluated by ELISA and have been expressed as O.D. at 450 nm in 1:160 serum dilutions. Significativity was estimated between Control Group A and the Test Group (● $P < 0.01$).

point, to reach a similar ELISA response, the plasma obtained by immunisation with the native M-CSF must be diluted 65-fold with respect to the one obtained from the animals immunised with the modified form. The separate estimation of IgG and IgM indicated that native M-CSF elicited a rapid and remarkable production of IgG but a low and rapidly decreasing amount of IgM. Low levels of both IgG and IgM antibodies were instead found with the PEG derivative.

Native PfChMab was found to induce in mice a delayed immunoresponse with respect to the native M-CSF. However, by repeated immunisation with this protein, a remarkable production of anti-PfChMab IgM was found while very low IgG levels were detected. Furthermore, the immunogenic profiles obtained with native PfChMab, PfChMab–PEG33 and PfChMab–mPEG19 were very similar (data not shown).

3.2. Tolerogenic properties of the monomethoxy-poly(ethylene glycol) derivatives

Fig. 2 reports the immunological time courses obtained by mouse pre-treatment with PEG conjugated M-CSF (Test Group), non conjugated PEG (Control Group A) and PBS only (Control Group B) and further sensitisation with native M-CSF.

Control Groups A and B displayed a similar behaviour ($P > 0.1$) indicating that PEG pre-administration did not affect the immunoresponse towards M-CSF. Nevertheless a remarkable difference in immunoresponse between the control groups and the test group was observed ($P < 0.05$). In particular, a lower production of anti-native M-CSF [IgG + IgM] was obtained in animals pre-treated with M-CSF–PEG with respect to the animals pre-treated with PEG or PBS. The separate estimation of anti-native M-CSF IgG and IgM indicated that M-CSF–PEG pre-administration did not influence the anti-M-CSF IgM generation while a relevant IgG suppression was observed (see Fig. 3). The data of Table 2, obtained by elaboration of the results of Fig. 3 according to the equation reported in literature [21,22], indicate that up to 87% suppression of anti-native M-CSF IgG was achieved by M-CSF–PEG pre-administration. Note that the suppressive effect of M-CSF–PEG was much higher when Freund's adjuvant was not used in the immunisation protocol.

The anti-native PfChMab IgM profiles obtained by pre-administration of PEG (Group 1), PfChMab–PEG19 (Group 2) and PfChMab–PEG33 (Group 3) to mice subsequently immunised with PfChMab are reported in Fig. 4. The time course behaviours demonstrate that pre-administration of the PEG conjugates induced a significant suppression of anti-native PfChMab IgM. Furthermore such suppressive effect was higher when the animals were pre-treated with PfChMab–PEG33. The data reported in Table 3 show that after the second immunisation the reduction of anti-PfChMab IgM was in the range of 75–94% when PfChMab–PEG33 was pre-administered, while it was of 50% when PfChMab–PEG19 was used. The data in Table 3 also indicate that the pre-administration of the PEG conjugates does not affect the anti-PfChMab IgG levels.

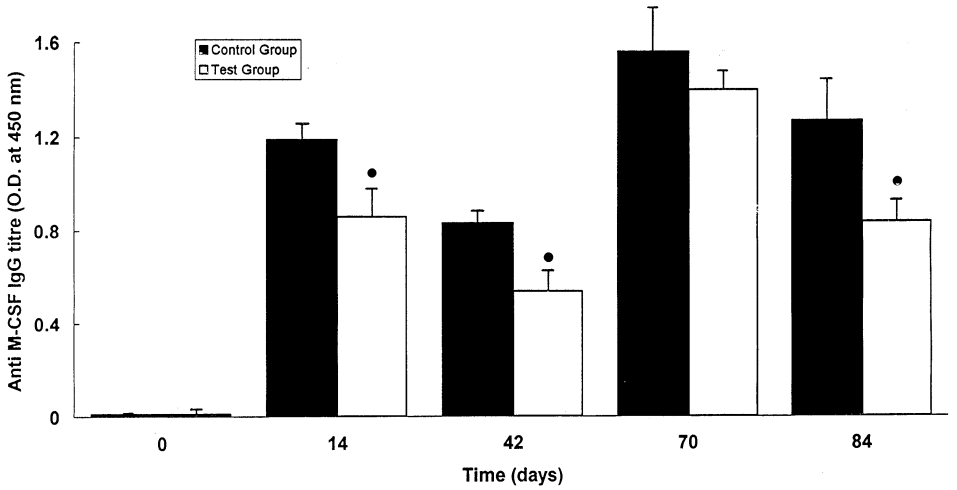

Fig. 3. Tolerogenic effect of M-CSF–PEG: suppression of anti-native M-CSF IgG production. Animals were pre-treated with PBS only (Control Group) and M-CSF–PEG (Test Group). The anti-M-CSF IgG levels were evaluated by ELISA and expressed as O.D. at 450 nm in 1:160 serum dilutions. Significativity was estimated between the Test and Control Groups (● $P < 0.005$).

Table 2
Suppression degree of anti-M-CSF IgG and IgM by pre-administration of M-CSF–PEG to mice

Days after the 1st immunisation	Suppression of anti-native M-CSF IgG (%)		Suppression of anti-native M-CSF IgM (%)	
	Immunisation with Freund's adjuvant	Immunisation without Freund's adjuvant	Immunisation with Freund's adjuvant	Immunisation without Freund's adjuvant
0	0	0	0	0
14	50	0	0	0
42	50	87	0	0
70	50	75	0	0
84	50	75	0	0

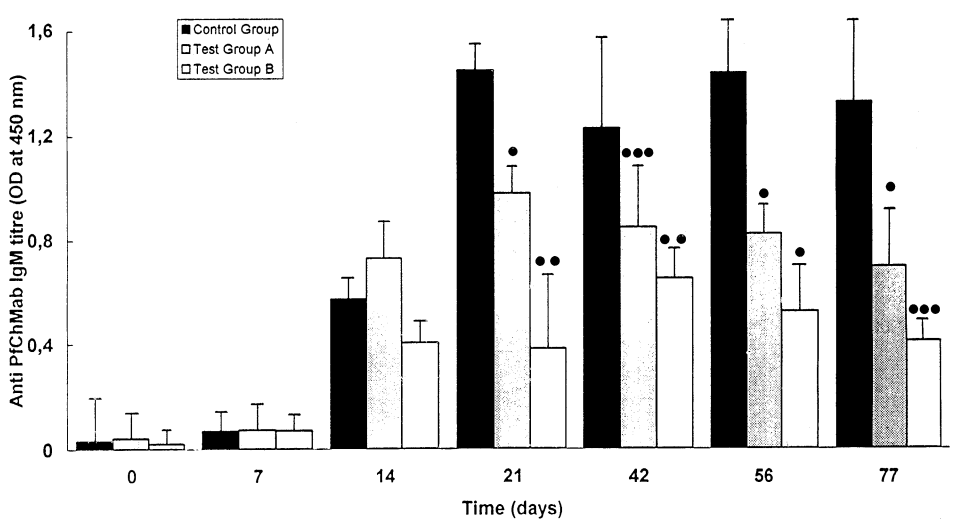

Fig. 4. Suppressive effect of PfChMab–PEG19 and PfChMab–PEG33 on anti-native PfChMab IgM production. Animals were pre-treated with PBS only (Control Group), PfChMab–PEG19 (Test Group A) and PfChMab–PEG33 (Test Group B). The anti-PfChMab IgM levels were evaluated by ELISA and expressed as O.D. at 450 nm in 1:160 serum dilutions. Significativity was estimated between Test Group A and the Control Group and between Test Groups A and B (● $P < 0.005$; ●● $P < 0.01$; ●●● $P < 0.05$).

Table 3
Suppression degree of anti-PfChMab IgM by pre-administration of PfChMab–PEG19 and PfChMab–PEG33 to mice

Days after the 1st immunisation	Suppression of anti-native PfChMab IgM (%)	
	Pre-treatment with PfChMab–PEG19	Pre-treatment with PfChMab–PEG33
0	0	0
14	0	0
21	50	94
42	50	75
56	50	87
77	50	87

4. Discussion

The modification of therapeutically active proteins by PEG attachment is known to change dramatically the immunological properties of this class of drugs. Many evidences reported in literature point out that PEGylation can significantly reduce the immunogenic potential of proteins and peptides and some others support the hypothesis that polymer coupling can switch an immunogenic compound into a tolerogenic one.

The data reported in the present investigation confirm the positive effect of PEG conjugation in enhancing the immunogenic properties of proteins. PEGylation, in fact, remarkably reduces the immunogenicity of M-CSF, a protein that possesses a relevant immunogenic character. However this effect can not be observed with PfChMab probably because this protein is characterised by an intrinsic low immunogenicity.

With regard to the tolerogenic characterisation of M-CSF and PfChMab, our data indicate that all the examined conjugates, M-CSF–PEG, PfChMab–PEG19 and PfChMab–PEG33, present tolerogenic character. Nevertheless the derivatives displayed a different immunosuppressive behaviour towards IgG and IgM production: the pre-administration of the M-CSF derivative reduced the generation of anti-native M-CSF IgG, but it did not affect the IgM production, while the pre-administration of the PfChMab–PEG derivatives reduced the anti-native PfChMab IgM production.

These findings suggest that, although polymer coupling can convey tolerogenic properties to immunogenic compounds, a possible relationship exists between the immunosuppressive performance of the conjugates and the immunological character of the native molecules. The different tolerogenic behaviour of the protein conjugates can in fact be related to the different capability of their native counterparts to elicit IgG and IgM. In particular the immunogenic profile obtained with M-CSF demonstrates that this protein possesses a relevant immunogenic potential, stimulating the production of high anti-native IgM levels soon after the second immunisation, whereas IgG generation takes place in a longer time. The rapid appearance of the primary immunoresponse could be the main reason of the PEG conjugate failure in inducing the tolerogenic effect towards the IgM production. In this case in fact only a reduction of anti-native M-CSF IgG was found. Instead in the case of PfChMab, that induces a slower IgM immunoresponse with respect to M-CSF, the conjugate was found to suppress efficiently the primary immunoresponse (IgM), while IgG production was apparently unaffected. To note that the low effect on IgG can be related to the slow production of this class of antibodies already observed with native PfChMab. Therefore, it is reasonable to think that the tolerogenic performance of polymer–immunogen conjugates is strictly related to the capability of the native molecule to elicit the immunoresponse in a suitable time.

The results on the tolerogenic potential of M-CSF–PEG obtained in presence or absence of the Freund's adjuvant further confirm the previous considerations. Although the adjuvant can not change the intrinsic immunological properties of the molecule, it can emphasise them by affecting both time and intensity of immunoresponse. Nevertheless, time and intensity of immunoresponse have been found to be involved in dictating the tolerogenic performance of the conjugate. Therefore, the use of the adjuvant can be reflected on the tolerogenic efficiency of the conjugate and in particular the lower immunosuppression observed with the protocol including the Freund's adjuvant can be due to a more rapid IgG immunoresponse.

With regard to PfChMab it is worth noting that, although preliminary competitive ELISA studies demonstrated that both of PEG conjugates do not recognise the specific antigen, these derivatives display a significant tolerogenic potential. This is in agreement with other studies reported in literature demonstrating that the maintenance of the biological activity of the immunogen does not represent a pre-requisite for the tolerogenic activity of the conjugate. On the other hand, the polymer composition of the construct seems to play a part in directing the tolerogenic efficiency of the polymer conjugated immunogens. The results obtained with the two PfChMab–PEG derivatives demonstrate that the suppression of the immunoresponse depends on the degree of modification of the tolerogen (number of polymer chains bound to the protein surface). In particular, the tolerogenic effect is significantly higher for the derivative of PfChMab modified with 33 PEG chains with respect to the derivative with 19 PEG molecules indicating that an optimal degree of modification should be achieved in order to obtain compounds with suitable tolerogenic properties.

5. Conclusions

The selective immunosuppression to an antigen by pre-administration of its polymer conjugate deals with the possibility to provide new pharmacological opportunities in long term therapies with immunogenic drugs [16,18]. This represents an interesting alternative to the traditional hyposensitisation treatments that are usually time-consuming methodologies, often ineffective and that can result in severe complications with the risk of anaphylactic response [17].

To date, many efforts have been made in order to elucidate the mechanisms that underlie this phenomenon and to understand the parameters that can affect the immunosuppression properties of the conjugates.

It now seems clear that the immunogenic character of the native molecule, as well as the degree of protein modification, play a role in dictating the tolerogenic properties of the conjugate, while the maintenance of the biological activity does not represents a pre-requisite for tolerogenic compounds.

However, the exploitation of effective and safe tolerisation procedures by using tolerogenic conjugates is a committing task that deserves of many accurate evaluations. The tolerogenic performance of the conjugates in fact depends upon a number of parameters that are still partially unknown and that must be carefully elucidate in order to avoid severe pharmacological complications.

References

[1] N.L. Burnham, Polymers for delivering peptides and proteins, Am. J. Hosp. Pharm. 51 (1994) 210–217.

[2] H. Maeda, T. Konno, Methamorphosis of neocarzinostatin to SMANCS: chemistry, biology, pharmacology and clinical effect of the first prototype anticancer polymer therapeutic, in: H.T. Maeda, K. Edo, N. Ishida (Eds.), Neocarzinostatin: The Past, Present and Future of an Anticancer Drug, Springer, Tokyo, 1997, pp. 207–267.

[3] T. Hirano, T. Todoroki, S. Kazo, H. Yamamoto, P. Caliceti, F.M. Veronese, H. Maeda, S. Ohashi, Synthesis of the conjugate of superoxide dismutase (SOD) with the copolymer of divinyl ether and maleic anhydride (DIVEMA) retaining enzymatic activity, J. Control. Rel. 28 (1994) 203–209.

[4] M. Morpurgo, O. Schiavon, P. Caliceti, F.M. Veronese, Covalent modification of mushroom tyrosinase with different amphiphilic polymers for pharmaceutical and biocatalysis applications, Appl. Biochem. Biotechnol. 56 (1996) 59–72.

[5] N.V. Kartre, Immunogenicity of recombinant IL-2 modified by covalent attachment of polyethylene glycol, J. Immunol. 144 (1990) 209–213.

[6] M.L. Nucci, R. Shorr, A. Abuchowski, The therapeutic value of poly(ethylene glycol)-modified proteins, Adv. Drug Del. Rev. 6 (1991) 131–133.

[7] N.V. Kartre, The conjugation of proteins with poly(ethylene glycol) and other polymers: altering properties of proteins and enhance their therapeutical potential, Adv. Drug Del. Rev. 10 (1993) 91–104.

[8] F.F. Davis, M. Kazo, M.L. Nucci, A. Abuchovski, Reduction of immunogenicity and extension of circulating half life of peptides and proteins, in: V.H.L. Lee (Ed.), Peptide and Protein Delivery, Marcel Dekker, New York, 1990, pp. 831–864.

[9] C. Delgado, G.E. Francis, D. Fisher, The uses of PEG-linked proteins, Crit. Rev. Ther. Drug Carr. Syst. 9 (1992) 249–304.

[10] P. Caliceti, O. Schiavon, L. Sartore, C. Monfardini, F.M. Veronese, Active site protection in the poly(ethylene glycol) surface modification of proteolytic enzymes, J. Bioact. Comp. Pol. 8 (1993) 41–50.

[11] P. Caliceti, M. Morpurgo, O. Schiavon, C. Monfardini, F.M. Veronese, Preservation of thrombolytic activity of urokinase modified with monomethoxypoly(ethylene glycol), J. Bioact. Comp. Pol. 9 (1994) 252–266.

[12] Y. Ashihara, T. Kono, S. Yamazam, Y. Inada, Modification of *E. coli* L-asparaginase with polyethylene glycol: disappearance of binding ability to anti-asparaginase serum, Biochem. Biophys. Res. Commun. 83 (1978) 385–391.

[13] W.Y. Lee, A.H. Sehon, Abrogation of reaginc antibodies with modified allergens, Nature 267 (1977) 618–622.

[14] W.Y. Lee, A.H. Sehon, E. Akerblom, Suppression of reaginic antibodies with modified allergens, Int. Arch. Allergy Appl. Immunol. 64 (1981) 100–114.

[15] A.H. Sehon, Suppression of antibody responses by conjugates of antigens and monomethoxypoly(ethylene glycol), Adv. Drug Del. Rev. 6 (1991) 203–217.

[16] T.P. King, L. Kochhoumien, N. Chiorazzi, Immunological properties of conjugates of ragweed pollen antigen E with monomethoxypolyethylene glycol or a co-polymer of D-glutamic and D-lysine, J. Exp. Med. 149 (1979) 424–428.

[17] Y. Chen, M. Takata, P.K. Maiti, S. Mohapatra, S.S. Mohapatra, A.H. Sehon, The suppressor factor of T suppressor cells induced by tolerogenic conjugates of ovalbumin and monomethoxypolyethylene glycol is serologically and physico–chemically related to the αβ heterodimer of the T cell receptor, J. Immunol. 152 (1994) 3–11.

[18] M.Z. Atassi, K.H. Ruan, K. Jinnai, M. Oshima, T. Ashizawa, Epitope-specific suppression of antibody response in experimental autoimmunoe myastenia gravis by a monomethoxypolyethylene glycol modified allergens, Proc. Natl. Acad. Sci. USA 89 (1992) 5852–5856.

[19] S. Bitoh, R. Wakefield, G.M. Lang, A.H. Sehon, Inhibition of the effector phase of IgE mediated allergies by tolerogenic conjugates of allergens and monomethoxypolyethylene glycol, Int. Arch. Allergy Immunol. 107 (1995) 316–318.

[20] G.M. Lang, A.H. Sehon, Analysis of T cell receptor αβ chains of CD8(+) suppressor T cells induced by tolerogenic conjugates of antigen and monomethoxypolyethylene glycol: involvement of TCR α CDR3 domain in immunosuppression, J. Immunol. 158 (1997) 1984.

[21] G.M. Lang, J.K. Jaszcuk, E.S. Rector, A.D. Milton, F. Emmrich, A.H. Sehon, Suppression of antibody response in mice to anti-murine anti-CD4 monoclonal antibodies by conjugates with monomethoxypolyethylene glycol, Immunol. Lett. 32 (1992) 247–252.

[22] S. Mohapatra, Y. Chen, M. Takata, S.S. Mohapatra, A.H. Sehon, Analysis of T-cell receptor αβ chains of CD8+ suppressor T cells induced by tolerogenic conjugates of antigen and monomethoxypolyethylene glycol, J. Immunol. 151 (1993) 688–698.

[23] L. Sartore, P. Caliceti, O. Schiavon, C. Monfardini, F.M. Veronese, Accurate evaluation of the polymer content in monomethoxypoly(ethylene glycol)-modified proteins based on amino acid analysis, Appl. Biochem. Biotechnol. 31 (1991) 213–221.

[24] B.A. Bidligmeyer, S.A. Cohen, T.L. Tarvin, Rapid analysis of aminoacids using pre-column derivatisation, J. Chromatogr. 336 (1984) 93–104.