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Two new murine monoclonal antibodies rised against human IgG

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Many pathological conditions are accompanied with changes in the concentration of the total IgG or some of its fraction. For this reason there is great interest in the production of reagents specific for IgG. In this paper, the binding characteristics of two new murine monoclonal antibodies (MoAb), assigned MoAb 15 and MoAb 22, are reported. These MoAbs were produced by hybridoma technology. By performing ELISAs and Western blots analyzes, it was demonstrated that both MoAbs interact specifically with human IgG. Cross reactivity with other sera proteins was not observed. In order to precisely localize the epitopes recognized by MoAb 15 and MoAb 22, the Western blots interactions of these MoAbs with electrophoreticaly separated IgG-fragments, obtained by the action of proteolytic enzymes (papain, pepsin, trypsin), were analyzed. According to the results of these experiments, both MoAbs interacted with epitopes in the C 3 domain. The affinity constants, calculated from Scatchard plots of binding of MoAb 15 and MoAb 22 to human IgG, were $Ka_{15} = 1.71\ \ 10^{6}\ M^{-1}$ and $Ka_{22} = 2.15\ \ 10^{9}\ M^{-1}$. According to all these findings, MoAb 15 and MoAb 22 could be used in standard immunochemical techniques. However, the experiments showed that both MoAbs had bad immunoprecipitating properties. In solid phase techniques (ELISAs, Western blot, dot-blot, *etc.*), their application gave excellent results that highly recommended them for use in these types of analyzes.

Keywords: human IgG, affinity constant, murine monoclonal antibodies, immunochemical technique.

INTRODUCTION

Hybridoma technology, designed by G. Kohler and C. Milstein in 1975,[1] is the oldest way to produce monoclonal antibodies (MoAb) with the desired binding characteristics. This protocol enabled the production of, theoretically, unlimited variety source of homogenous amounts of MoAbs with "predefined" characteristics of binding (affinity and specificity). In spite of the development of new technologies based on methods of genetic engineering,[2] this procedure is still widely used.

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After the appearance of hybridoma technology, MoAbs became the "reagents of choice" in research and diagnostic. The application of MoAbs made standard procedures more specific and more sensitive.[3] However, each application of MoAbs required its detailed characterization as a guarantee for the validity of the obtained results. So, expression "to produce MoAb-reagents" meant to obtain a MoAb and to fully characterize it.

Our goal was the production of MoAbs specific for human IgG (hIgG). Interest in the production of reagents specific for IgG can be explained by the fact that many pathological conditions are accompanied by changes in the concentration of the total IgG or some of its fractions.[4]

In this paper, investigations on the binding characteristics of two new murine MoAbs produced by hybridoma technology, assigned MoAb 15 and MoAb 22, are described. The investigations showed that they reacted specifically with hIgG, a fact that could make them usefull, primarily as diagnostic tools.

EXPERIMENTAL

Determination of affinity of MoAb 15 and MoAb 22 for hIgG by competitive ELISA

Affinity constants (*K*a) of MoAb 15 and MoAb 22 were calculated, according to Scatchard analyze, from the results obtained by competitive ELISA. In this experiment, a constant amount (1 g/ml *i.e.,* 6.4 nM) of MoAb labeled by biotin (MoAb-B) was incubated in the presence of different concentrations of hIgG for 2 h at 25 ºC. Determination of the concentrations of free MoAb (F) in these samples were based on binding to hIgG adsorbed on microtitar plate, while the concentrations of MoAb 15 and MoAb 22 bound to hIgG in solution (B) were equal to the differences between the total MoAb concentration and F.

For determination of F_{15} and F_{22}, hIgG was adsorbed on microtitar plates from hIgG/PBS solution at concentrations of 0.5 g/ml and 1 g/ml, respectively (50 l/well, at 4 ºC, over night). A solution of 1 % BSA (Sigma)/PBS was used for saturation (200 l/well, 2 h, at room temperature), preparation of samples and dilution of streptavidin-peroxidase (ICN) (50 l/well, 1 h, 25 ºC). Before each step, following saturation, the plates were washed with 0.05 % Tween 20 (Sigma)/PBS (4 200 l/well) and PBS (1 l/well). OPD (Sigma) (50 l/well) was used as substrate and its transformation by streptavidin-peroxidase was stopped by adding 2 M H_2SO_4 (50 l/well). The amount of MoAb-B bound to hIgG adsorbed on the plates was proportional to the absorbance read at 492 nm.

Fragmentation of hIgG by:

a) papain

Fragmentation of hIgG by papain was performed in 0.1 M Na-phosphate buffer, pH 6.5, in the presence of 50 mM Cys (Merck) and 1 mM EDTA (Fluka). The final ratio of masses of hIgG and papain (Gibco BRL) ($m_{hIgG} : m_{papain}$) was 100 : 1. The digestive mixture was incubated at 37 ºC for 16 h. The reaction was stopped by adding a solution of iodacetamide to a final concentration of 75 mM.

b) pepsin

Fragmentation of hIgG by pepsin was performed in 0.1 M acetic buffer, pH 3.5, at 37 ºC. The ratio of the amount of hIgG and pepsin (Sigma) in the digestion mixture was $m_{hIgG} : m_{pepsin} = 100 : 1$. The reaction was stopped in intervals of 1 h by raising the pH to 9.

c) trypsin

Fragmentation of the isolated Fc portion of hIgG (hFc) by trypsin was performed in 10 mM Tris / 0.1 M NaCl, pH 7.8, at 40 ºC for 1 h. Before the addition of trypsin, the pH of the hFc solution

was lowered to 2.5 by adding 2 M HCl. The solution was incubated for 5 min and the pH was returned to 7.8 by adding 1 M Tris. The ratio of hFc and trypsin in the reaction mixture was m_{hFc} : $m_{trypsin}$ = 100 : 2.

RESULTS AND DISCUSSION

Murine hybridoma 15 and 22, which secreted MoAb 15 and MoAb 22, respectively, were obtained by fusion of murine mieloma cells SP/02 mIL-6 and spleen cells of BALB/c mouse immunized with hIgG. Preliminary tests, performed with supernatant of clones 15 and 22, indicated that MoAb 15 and MoAb 22 interacted with hIgG. Further investigations, performed with MoAb 15 and MoAb 22 isolated from appropriate ascitic fluids, confirmed this observation.

Affinity of MoAb 15 and MoAb 22 for hIgG

The affinity of MoAb for its Ag is one of the most important properties determining its usefulness.[5] The affinities of MoAb 15 (Ka_{15}) and MoAb 22 (Ka_{22}) for hIgG were determined by competitive ELISA. In these experiments, the binding of MoAb 15 and MoAb 22 (both at constant concentration, 1 g/ml) to hIgG adsorbed on microtitar plate were inhibited by incubation with different amounts of hIgG. The obtained results

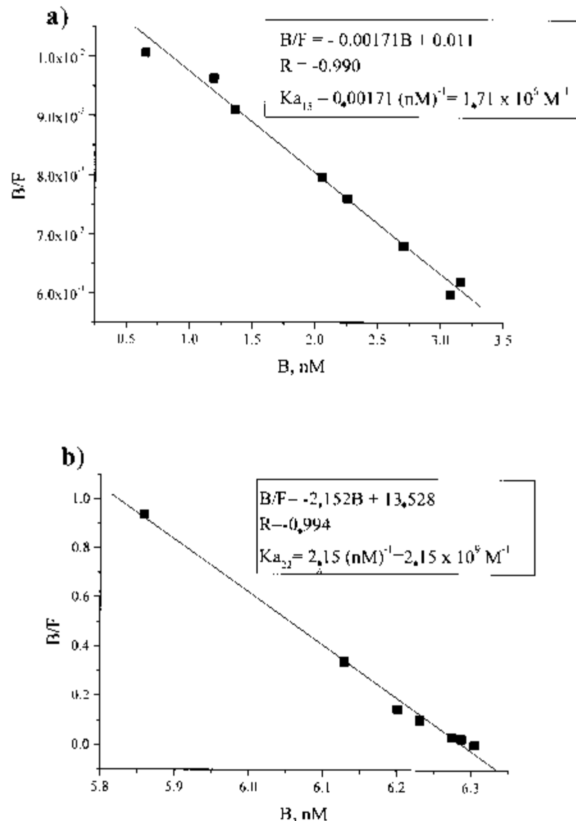

Fig. 1. Scatchard plots ($B/F = f(B)$) for the binding of MoAb 15 a) and MoAb 22 b) to hIgG; B – concentration of MoAb bound to hIgG, F – concentration of free MoAb.

were transformed according to the Scatchard equation and the value of *K*a was determined from Scatchard plot (Fig. 1). *K*a values of MoAb 15 and MoAb 22 for hIgG are: $Ka_{15} = 1.71 \quad 10^6\ M^{-1}$ and $Ka_{22} = 2.15 \quad 10^9\ M^{-1}$, respectively.

According to its affinity for hIgG, MoAb 22 might be used, after coupling to an inert matrix, for the isolation of hIgG by affinity chromatography, ELISA, Western blot, dot-blot, immunofluorescence and in different forms of immunoprecipitation.[5] However, MoAb 22, despite of high affinity, had bad immunoprecipitation characteristics and did not precipitate hIgG either in solution or in agarose gel. Steric hindrances or functional monovalency[6] that would prevent the formation of an immunoprecipitation lattice could be the explanations of this phenomenon.

The Ka_{15} value of MoAb 15 implied that it could be used in tests such as ELISA and Western blot, too.[5] However, it also showed bad immunoprecipitation properties, most probably, because of its low affinity for hIgG.

Localization of the epitopees recognized by MoAb 15 and MoAb 22

Western blot analyzes of electrophoreticaly separated human sera proteins, showed that both MoAbs reacted with the heavy chain of human immunoglobulins (Fig. 2). According to the results of these experiments, the reactivity of MoAb 15 and MoAb 22 with other, "non-IgG", sera proteins could also be excluded. The non-existence of cross-reactivity is very important if these MoAbs are to be used for the quantification of hIgG.

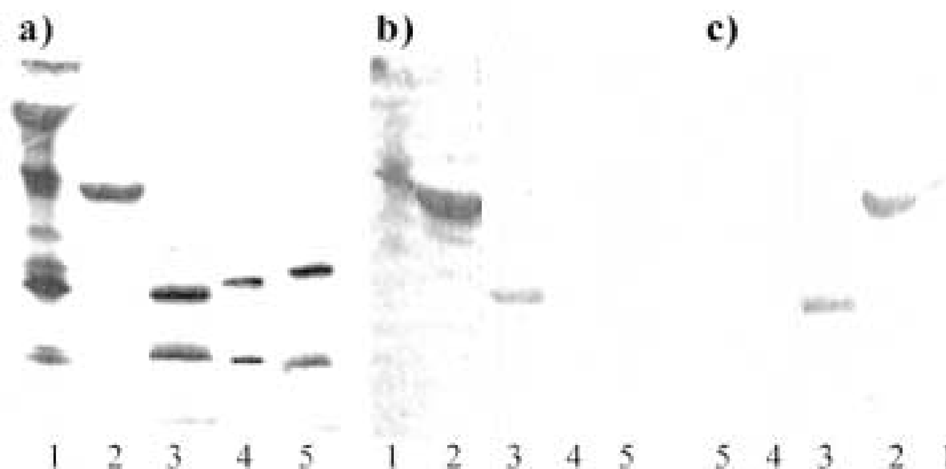

Fig. 2. SDS-PAGE in 4 – 15 % PAAG a) and Western blot analyzes of 1 – human sera, 2 – hIgG, 3 – hIgG reduced with -mercaptoethanol, 4 – hIgA reduced with -mercaptoethanol 5 – hIgM reduced with -mercaptoethanol; blots were developed in presence of MoAb 22 (b) and MoAb 15 (c).

The determination of the concentration of hIgG is usually performed by different types of ELISAs. We showed that both MoAbs could be used successively in these assays. Using MoAb 15 or MoAb 22, less than 1 g/ml of hIgG could be detected by

ELISA. The low value of the additivity index (*AI*)[7] for MoAb 15 and MoAb 22, $AI_{15,22}$ = 6.22, indicated that these MoAbs, because of steric hindrance for the simultaneous binding on hIgG, could not be used in sandwich ELISA which is more specific than others types of ELISAs. In "sandwich" ELISA they could be used in combination with MoAbs that recognize epitopes located far from those of MoAb 15 and MoAb 22. For example, they could be used with MoAb 44 (specific form human κ chain) for the determination of the concentration of IgGκ molecules.

In order to localize the epitopes recognized by MoAb 15 and MoAb 22 more precisely, the interactions of these MoAbs with fragments of hIgG, obtained by the action of papain, trypsin and pepsin, were analyzed.

The main products of papain digestion of hIgG were fragments of equal molecular size (50 kDa), Fab and Fc.[8] They could be separated by affinity chromatography on protein A.[9] The fragments obtained by papain digestion were analyzed by Western blot. According to the bands on blots of sample eluted from protein A, which appeared in the presence of both MoAbs (Fig. 3), it was concluded that the epitopes recognized by MoAb 15 and MoAb 22 were located in the Fc portion of hIgG.

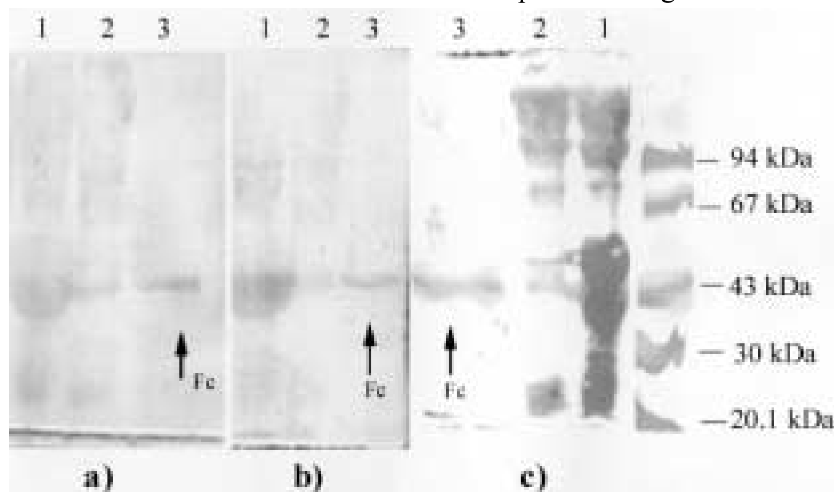

Fig. 3. SDS-PAGE (7.5 % PAAG) of hIgG fragments obtained by the action of papain (c) and Western blot analyses of the interaction of MoAb 15 (a) and MoAb 22 (b) with them; samples: 1 – digestive mixture of hIgG resulting from the action of papain, 2 – fraction of the digestive mixture of hIgG resulting from the action of papain which did not interact with protein A, 3 – fraction of digestive mixture of hIgG resulting from the action of papain which was eluted from protein A by 0.1 M citric buffer, pH 3.5.

It is well known that peptic digestion of hIgG yields $(Fab)_2$ (100 kDa) and pFc' as the main products. Beside these fragments, numerous intermediate products and peptides resulting from the enzymatic degradation of C 2 occur in the digestion mixture[10] (Fig. 4a). Four major bands appeared on blots developed in the presence of MoAb 15 (Fig. 4b) or MoAb 22 (Fig. 4a), after electrophoretical separation of the hIgG

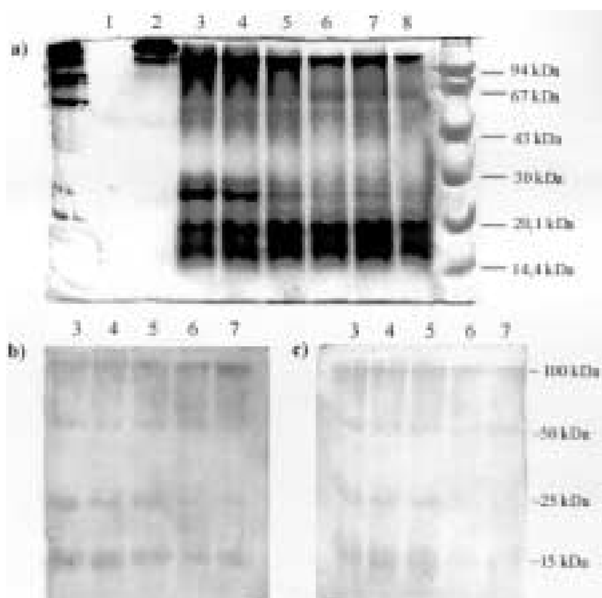

Fig. 4. SDS-PAGE (10 % PAAG) of hIgG fragmentized with pepsin (a) and Western blot analyzes of the interactions of MoAb 15 (b) and MoAb 22 (c) with the obtained fragments; samples: pepsin at concentration used for digestion (1), intact hIgG (2), mixtures of hIgG fragments obtained by the action of pepsin for 1 h (3), 2 h (4), 3 h (5), 4 h (6), 6 h (7) and 8 h (8).

fragments obtained by the action of pepsin. These bands, assigned according to corresponding molecular weights, were: " 100 kDa", " 50 kDa", " 25 kDa" and " 15 kDa". The positions of molecular weights markers in the polyacrylamide gel (PAAG) indicated that, in this system, resolution of protein with "higher" (>100 kDa) molecular weight was not correct (Fig. 4a). So, it is possible that " 100 kDa" bands on blots were the result of interaction of MoAb 15 or MoAb 22 with intact hIgG or its intermediates that possesed a partially degraded Fc portion. Regarding the fact that the major cleavage site of pepsin is in the lower hinge region, below interchain disulphide bonds,[10] the appearance of " 50 kDa" in the PAAG was unexpected. It is possible that this band was the result of stochastic formation of disulphide bonds between free Cys residues of fragments produced by the action of pepsin. Interactions with proteins of band " 25 kDa" (C 2-C 3) confirmed our finding that epitopes recognized by MoAb 15 and MoAb 22 were located in the Fc portion of hIgG.

Interactions of MoAb 15 and MoAb 22 with peptic bands of lower molecular weight indicated that epitopes recognized by these MoAbs could be located in the C 3 domain of the human chain. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyzes of fragments resulting from tryptic digestion of the hFc portion of Ig confirmed this hypothesis. HFc were obtained by papain digestion of hIgG, isolated by affinity chromatography on protein A and exposed to low pH (pH 2.5). Exposure to low pH rendered the region between C 2 and C 3 domains transiently susceptibile to trypsin upon return to neutral pH, allowing the splitting of Fc to those

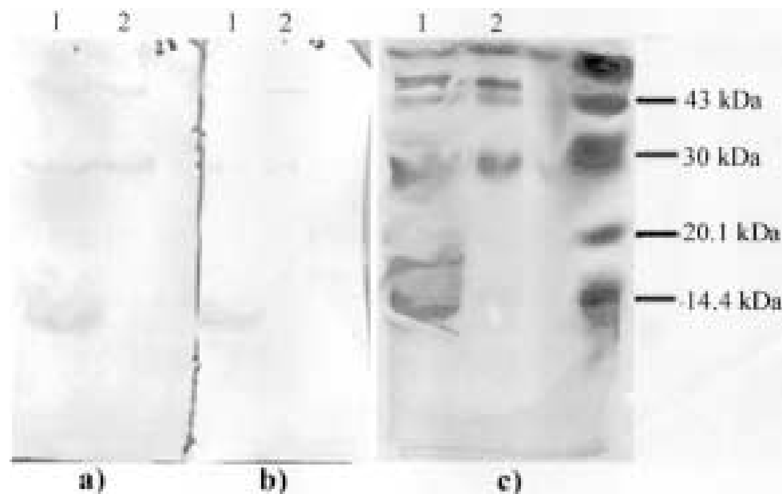

Fig. 5. SDS-PAGE (15 % PAAG) of hFc fragments obtained by the action of trypsin (c) and Western blots analyzes of the interaction of MoAb 15 (b) and MoAb 22 (a) with these fragments; 1 – hFc fragmentized by trypsin, 2 – hFc exposed to low pH.

two domains.[11] After SDS-PAGE of tryptic digestion mixture, there were 3 bands in PAAG: 25 kDa, 17 kDa and 12.5 kDa (Fig. 5a). Western blot analyzes showed that both MoAbs reacted with the " 25 kDa" band (unseparated between C 2-C 3) and the band of the lowest molecular weight (Fig. 5b, c) which, according to literature data, represents the C 3 domains.[12]

Precise localization of epitopes recognized by a MoAb could be useful data in functional studies, inhibition studies or in designing experiments that require simultaneous use of more MoAbs.

CONCLUSION

MoAb 15 and MoAb 22, obtained by hybridoma technology, were specific for hIgG. They specifically interacted with epitopes localized in the C 3 domain of the human chain. The affinity of the interaction of MoAb 22 was high (Ka_{22} = 2.15 10^9 M^{-1}), while MoAb 15 bound hIgG with low affinity (Ka_{15} = 1.71 10^6 M^{-1}). According to our results, MoAb 15 and MoAb 22 did not precipitate hIgG either in solution or in agarose gel. This inability to form precipitating complexes with hIgG reduce their applicability. Otherwise, both MoAbs, when used independently, demonstrated excellent properties in common diagnostic tests such as ELISA, blots, *etc*. However, in these assays advantage could be given to MoAb 22 because of its greater affinity that allows the detection of hIgG at lower concentrations.

И З В О Д

ДВА НОВА МИШЈА МОНОКЛОНСКА АНТИТЕЛА ДОБИЈЕНА ПРОТИВ ХУМАНОГ IgG-а

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Многа патолошка стања су повезана са променама концентрације укупног IgG-а или неке од његових фракција. То је разлог великог интересовања за продукцију реагенаса специфичних за IgG. Ми смо у овом раду описали карактеристике везивања два нова мишја моноклонска антитела (MoAt), означена као MoAt 15 и MoAt 22. Ова MoAt су добијена хибридомском технологијом. Користећи ELISA-е и Western blot анализе, показали смо да оба MoAt специфично реагују са хуманим IgG-ом. Укрштена реактивност са другим серумским протеинима није уочена. Да би смо прецизно лоцирали епитопе које препознају MoAt 15 и MoAt 22, Western blot-ом смо анализирали интеракције ових MoAt са електрофоретски раздвојеним фрагментима IgG-а добијених дејством протеолитичких ензима (папаин, пепсин, трипсин). Према резултатима ових експеримената оба MoAt интерреагују са епитопима у C 3 домену. Константе афинитета, израчунате са Скачардових дијаграма везивања MoAt 15 и MoAt 22 за хумани IgG су $Ka_{15} = 1.71 \;\; 10^{6}\ \mathrm{M}^{-1}$ и $Ka_{22} = 2.15 \;\; 10^{9}\ \mathrm{M}^{-1}$. На основу свих ових чињеница, MoAt 15 и MoAt 22 би се могла користити у стандардним имунохемијским техникама. Међутим, наши експерименти су показали да оба MoAt имају лоше имунопреципитационе особине. Са друге стране, у техникама на чврстој фази (ELISA-е, Western blot, dot-blot, итд.) њихова примена је дала одличне резултате што их препоручује за употребу у овом типу анализа.

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REFERENCES

1. G. Kohler, C. Milstein, *Nature* **256** (1975) 495
2. P. J. Hundson, *Current Opinion in Immunol.* **11** (1999) 548
3. C. K. Borrebaeck, *Imm. Today* **21** (2000) 379
4. A. K. Abbas, A. H. Lichtman, J. S. Pober, *Cellular and Molecular Immunology*, W. B. Saunders Co., 2nd ed. 1994
5. E. Harlow, D. Lane, *Antibodies - a Laboratory Manual*, Coald Spring Harbor Laboratory, 1988
6. R. A. Margni, R. A. Binaghi, *Ann. Rew. Immunol.* **6** (1988) 535
7. T. E. Creighton, *Protein Structure - a Practical Approach*, IRL Press, 1989
8. F. A. Nardella, D. C. Teller, *Mol. Immunol.* **22** (1985) 705
9. J. W. Goding, *J. Immunol. Methods* **20** (1978) 241
10. M. W. Turner, H. H. Bennich, J. B. Natvig, *Nature* **225** (1970) 853
11. C. Endersen, M. Heggeness, A. Grov, *Scand. J. Immunol.* **3** (1973) 261
12. J. R. Ellerson, D. Jasmeen, R. H. Painter, K. J. Dorrington, *J. Immunol.* **116** (1976) 510.