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Antibodies Against Cerebral M₁ Cholinergic Muscarinic Receptor from Schizophrenic Patients: Molecular Interaction

Tania Borda,*† Ricardo Perez Rivera,* Lilian Joensen,† Ricardo M. Gomez,*† and Leonor Sterin-Borda2*†

We demonstrated the presence of circulating Abs from schizophrenic patients able to interact with cerebral frontal cortex-activating muscarinic acetylcholine receptors (mAChR). Sera and purified IgG from 21 paranoid schizophrenic and 25 age-matched normal subjects were studied by indirect immunofluorescence, flow cytometry, immunoblotting, dot blot, ELISA, and radioligand competition assays. Rat cerebral frontal cortex membranes and/or a synthetic peptide, with an amino acid sequence identical with that of human M₁ mAChR, were used as Ags. By indirect immunofluorescence and flow cytometry procedures, we proved that serum-purified IgG fraction from schizophrenic patients reacted to neural cell surfaces from rat cerebral frontal cortex. The same Abs were able to inhibit the binding of the specific M₁ mAChR radioligand [³⁵S]pirenzepine. Immunoblotting experiments showed that IgG from schizophrenic patients revealed a band with a molecular mass coincident to that labeled by an anti-M₁ mAChR Ab. Using synthetic peptide for dot blot and ELISA, we demonstrated that these Abs reacted against the second extracellular loop of human cerebral M₁ mAChR. Also, the corresponding affinity-purified antipeptide Ab displayed an agonistic-like activity associated to specific receptor activation, increasing cyclic GMP production and inositol phosphate accumulation, and protein kinase C translocation. This paper gave support to the participation of an autoimmune process in schizophrenia. The Journal of Immunology, 2002, 168: 3667–3674.

Schizophrenia is a brain disease of unknown etiology, with a lifetime incidence of at least 1% of the general population. Numerous theories have been formulated and tested, and they continue to compete for pride of place as the essential explanation for why patients suffer from periodic episodes of psychotic symptoms and remissions and typically decline in social and cognitive functions, i.e., the schizophrenia syndrome. The occurrence of schizophrenia has been ascribed to multiple etiopathological factors including psychodynamic, neurological, genetic, environmental, and immunological influences.

One scientific approach is based on the hypothesis that the immune system or specific immunological dysregulation could be involved in the manifestation of this psychotic disorder. Both the unspecific and the specific arms of the immune system seem to be involved in the immune dysfunction of schizophrenia (1). The unspecific, “innate” immune system shows signs of overactivation in unmedicated schizophrenic patients, as indicated by increased monocytes and γδ T cells. Increased levels and activation of IL-6 might be the result of the activation of monocytes/macrophages. In contrast, several parameters of the specific cellular immune system are blunted, such as the decreased Th1-related immune parameters in schizophrenic patients both in vitro and in vivo. It seems an association between Th1/Th2 imbalances with a shift to the Th2 system in schizophrenia (1).

Evidence pointing to a role for autoimmune comes from several sources. Heath and colleagues (2, 3) were probably some of the first proposing that anti-brain humoral factors (Abs) might be involved, producing some schizophrenic manifestations (4). Since then, much evidence have been accumulated showing circulating Abs against central nervous tissue and other Ags, indicating an autoimmune basis for schizophrenia (3, 5–11).

However, with the introduction of neuroleptics into schizophrenic therapy, the focus of interest moved away from the immune system, leading to the dopamine hypothesis as the center for research activities (1). Although a primary disturbance in dopamine function in schizophrenia cannot be ruled out, the intimate relationship between dopaminergic and other neuronal systems must be emphasized (12, 13). One cannot be disregard the possible involvement of other amine, amino acid, or peptide transmitters (13).

Considerable evidence demonstrates extensive interactions between the dopamine and the cholinergic system (14, 15). Moreover, clinical, pharmacological, and anatomical findings evidenced a role of cholinergic neurons in the pathology of schizophrenia (13, 16). Hence, there is a well-documented association between muscarinic activity and the modulation of certain CNS functions altered in schizophrenia, such as executive functions, memory, attention, and motor control (17–19). The role of muscarinic receptor is further supported by the actions of clozapine and olanzapine, drugs that bind with high affinity to muscarinic receptors, suggesting that the muscarinic system is more relevant to their greater efficacy than conventional neuroleptics, indeed efficient in the treatment of schizophrenia (18, 20, 21).

Moreover, it has been proposed that muscarinic cholinergic neurotransmission may be increased in schizophrenia (17, 20, 22). The high number of cholinergic projections in human cortex suggests a potential role in the regulation of sensory afferents (23). Also, muscarinic M₁, M₂, and M₄ receptors have been found in high density in human frontal cortex (24). The M₁ subtype receptor,
in particular, may be involved in modulation of excitatory amino acid neurotransmission in cortical and limbic areas (25).

On the basis of the autoimmune hypothesis of schizophrenia, we focused our research on the possibility of CNS-specific Ag-Ab system in this psychiatric condition. We investigated the acetylcholine neurotransmitter system, screening sera from schizophrenic patients for autoantibodies against the neurotransmitter muscarinic acetylcholine receptors (mACHR).3 An immunological influence on this system could result in a functional dysregulation, thereby triggering, in interaction with other factors, the pathophysiology of psychotic symptoms.

Materials and Methods

Subjects

The studied group comprised 21 inpatients (15 men, 6 women) suffering from paranoid schizophrenia (n = 18; mean age, 44 ± 8 years; range, 25–56 years; mean duration of illness, 26 ± 7) as defined by DSM-IV criteria (26). Patients did not have any associated organic disease and, more specifically, no immune disorders. All patients were receiving maintenance doses of neuroleptic medication. Diagnoses were made on the basis of clinical records, interview data collected with the Structured Clinical Interview for DSM-IV (27) and the Brief Psychiatric Rating Scale (28) (schizophrenic score was 46.5; SD = 15.1; predominance of negative symptoms). The control group consisted of 25 age-matched healthy subjects (15 men, 10 women; mean age, 45 ± 5 years). Patients and volunteers gave their expressed consent to participate in this study. All clinical investigations have been conducted according to the principles expressed in the Helsinki Declaration.

Peptides

A 24-mer peptide, ERTGAGGCYIQFSEQRHHTFGTM, corresponding to the amino acid sequence of the second extracellular loop of the human M1 mAChR was synthesized by Fmoc-amino acids activated using 1-hydroxybenzotriazole/dicyclohexylcarbodiimide strategy with an automatic peptide synthesizer (Model 431A; Applied Biosystems, Foster City, CA). The peptide was desalted, purified by HPLC, and subjected to N-terminal sequence analysis by automatic Edman degradation with an Applied Biosystems 470A sequencer.

Purification of human IgG

The serum IgG fraction from normal and schizophrenic subjects was isolated by protein G affinity chromatography as described elsewhere (29) for protein A and standardized for protein G. Briefly, sera were loaded onto the protein G (Sigma-Aldrich, St. Louis, MO) affinity column equilibrated with 1 M Tris-HCl, pH 8.0, and the columns were then washed with 10 volumes of the same buffer. IgG fraction were eluted with 100 mM glycine-HCl, pH 3.0, and immediately neutralized. Both IgG concentration and purity were determined by radial immunodiffusion assay. The IgG purification was critical for the specificity of Ab effect, because it abolished serum neuroleptic medication actions.

Purification of antipeptide Abs by affinity chromatography

The IgG fraction of 20 schizophrenic patients was independently subjected to affinity chromatography on the synthesized peptide covalently linked to Affi-Gel 15 gel (Bio-Rad, Richmond, CA). The IgG fraction was loaded on the affinity column equilibrated with PBS, and the nonpeptide fraction was first eluted with the same buffer. Specific antipeptide autoantibodies were then eluted with 3 M KSCN, 1 M NaCl, followed by immediate extensive dialysis against PBS. The IgG concentration of both nonantipeptide Abs and specific antimuscarinic receptor peptide Abs were determined by radial immunodiffusion assay, and their immunological reactivity against the muscarinic receptor peptide was evaluated by ELISA (30).

Rat cerebral frontal cortex membrane preparations

Female Wistar rats (obtained from the Pharmacology Unit, School of Dentistry, University of Buenos Aires, Buenos Aires, Argentina) were housed in our colony in small groups and kept in automatically controlled lighting (lights on 8 a.m.–7 p.m.) and uniform temperature (25°C) conditions. All

animals were used at 3–4 mo of age. The animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care. Membranes from cerebral frontal cortex were prepared as previously described (31). In brief, tissues were homogenized in an Ultra-Turrax at 4°C in 5 volumes of 10 mM potassium phosphate buffer, 1 mM MgCl₂, 0.25 M sucrose (buffer A), pH 7.5, supplemented with 0.1 mM PMSF, 2 μg/ml leupeptin, and 1 μM pepstatin A. The homogenate was centrifuged twice for 10 min at 3000 × g, then at 10,000 × g, and 15,000 × g at 4°C, for 15 and 90 min, respectively. The resulting pellets were resuspended in 50 mM phosphate buffer with the same protease inhibitors pH 7.5 (buffer B).

Indirect immunofluorescence technique

Newborn rat frontal brains were harvested and mechanically dissociated, and aliquots were cytoplasm onto slides. Cell slides were incubated for 60 min with schizophrenia or normal IgG (1 × 10⁻⁶ M) at room temperature in a wet chamber. After three washings with PBS, cells were further incubated with rabbit anti-human IgG FITC-conjugated F(ab)² (1/100) (DAKO, Glostrup, Denmark) for 30 min at room temperature in a humidified chamber. After three additional washes with PBS, slides were mounted in PBS-glycerol and observed with a Nikon photomicroscope equipped with epi-illumination (32).

Measurement of IgG binding on neural cells by flow cytometry

Neural cells were obtained as described above. After cells were washed with PBS (Ca²⁺ and Mg²⁺ free), pellets were resuspended in PBS containing IgG (1 × 10⁻⁶ M) from schizophrenic patients or normal subject as negative control. After 1-h incubation at 4°C, cells were washed and further incubated for 30 min with rabbit anti-human IgG FITC-conjugated F(ab)² (1/100). Cells were then fixed with 1% paraformaldehyde and analyzed by flow cytometry in a FACScan cytomicroscope (BD Biosciences, San Jose, CA). Fluorescence attributable to FITC-conjugated Abs was excited by an argon laser operating at 488 nm. Emission from fluorescein was measured using bandpass filters at 525 nm. Appropriate settings of forward and side scatter gates were used to examine 10,000 cells per experiment. The percentage of positive cells was determined by the thresholds set with isotopic controls. The numbers of fluorescent molecules per cell were indirectly measured by assessing the mean intensity of arbitrary units of fluorescence of cells.

Immunoblotting assay

Rat cerebral frontal cortex membranes were subjected to electrophoresis on 8.5% SDS-polyacrylamide gels as previously described (30). Proteins were transferred to nitrocellulose sheets and revealed with different IgG from schizophrenic or normal individuals. As a control, we also tested an anti-M₁ mAChR Ab from Research and Diagnostic Antibodies (Berkeley, CA). Briefly, each lane was incubated with 1 × 10⁻⁸ M control M₁ mAChR IgG or anti-M₁ peptide IgG from schizophrenic patients in the presence or absence of 1 × 10⁻⁸ M synthetic peptide or normal IgG in 25 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS) for 2 h at room temperature, washed extensively with TBS-Tween (0.05%), and incubated with goat anti-human IgG-alkaline phosphates conjugate (1/10,000) (Sigma-Aldrich) for 1 h before 5-Bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium were added. Molecular mass standards run in parallel were myosin (205 kDa), β-galactosidase (140 kDa), BSA (83 kDa), carbonic anhydrase (45 kDa), and soybean trypsin inhibitor (33 kDa) from Bio-Rad.

Dot blot assay

Nitrocellulose discs were dotted with 2 μg M₁ mAChR synthetic peptide. Then they were diluted in TBS and blocked with TBS-5% skim milk and incubated with a 2-fold dilution of sera from schizophrenic or normal subjects or its corresponding IgG (1 × 10⁻⁶ M) alone or preincubated with 1 × 10⁻⁸ M M₁ mAChR synthetic peptide in TBS-5% skim milk for 2 h at room temperature. After three washes with TBS-0.05% Tween 20, the immune complexes were revealed with alkaline phosphatase-labeled goat anti-human Ig (Sigma) (1/10000 dilution) followed by the addition of the chromogenic substrate mixture nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate at a 1:1 ratio under alkaline conditions (32).

Radioligand binding assay

Receptor ligand binding was performed as described previously (31) Aliquots of the membrane suspension (50 μg protein), pretreated or not with increasing concentrations of IgG from normal or schizophrenic subjects for 30 min at 30°C, were incubated with increasing concentrations of [³H]pirenzepine (New England Nuclear; sp. act. 85.6 Ci/mmol) for 60 min at 25°C in a total volume of 150 μl buffer B. Binding was stopped by
adding 2 ml ice cold buffer followed by rapid filtration (Whatman GF/C filter; Whatman, Maidstone, U.K.). Filters were rinsed with 12 ml ice cold buffer, transferred into vials containing 10 ml scintillation mixture, and counted in a liquid scintillation spectrometer. Nonspecific binding was determined in the presence of 10⁻³ M atropine (Sigma-Aldrich) and never exceeded 10% of total binding. Radioactivity bound was lower than 10% of total counts.

**ELISA**

Fifty microliters of peptide solution (20 μg/ml) in 0.1 M Na₂CO₃ buffer, pH 9.6, were used to coat Costar microtiter plates at 4°C overnight. After the wells were blocked with 2% BSA in PBS for 1 h at 37°C, 100 μl 1/10 dilution of sera or 1 μg/ml purified IgG from schizophrenic or normal subjects were allowed to react with peptide for 2 h at 37°C. Wells were then thoroughly washed with 0.05% Tween in PBS and 100 μl 1/6000 goat anti-human IgG-alkaline phosphatase-conjugated Abs (Sigma-Aldrich) were added and incubated for 1 h additionally at 37°C. After extensive washings, p-nitrophenyl phosphate (1 mg/ml) was added as substrate. After 30 min, OD values were measured at 405 nm with an ELISA reader (Uni-skan Labsystem) (30). As negative controls non-Ag paired wells and wells with no primary antiserum, were done.

**Cyclic GMP (cGMP) assay**

Rat cerebral frontal cortex slices (0.3 g) were incubated in 1 ml Krebs-Ringer bicarbonate buffer (KRB) containing 0.1 mM isobutylmethylxanthine for 30 min under a constant current of 5% CO₂ in O₂. IgG or pilocarpine was added at the last 10 min, whereaspirenzepine was included in the incubation volume from the beginning. Reactions were started by homogenization as previously stated (30). Samples were assayed by RIA using²⁻¹⁻labeled cGMP (DuPont New England Nuclear, Wilmington, DE; 2200 Ci/mmol) and anti-cGMP antiserum (Sigma-Aldrich).

**Measurement of total labeled inositol phosphates (IPs)**

Rat slices from cerebral frontal cortex were incubated for 120 min in 0.5 ml of KRB gassed with 5% CO₂ in O₂ with 1 mCi [myo⁻³H]inositol (sp. act. 15 Ci/mmol; DuPont New England Nuclear, Wilmington, DE; 2200 Ci/mmol) and by anti-cGMP antiserum (Sigma-Aldrich).

**Protein kinase C (PKC) activity assay**

PKC activity was assayed on both cytosolic and membrane preparations by measuring the incorporation of³²P from [γ⁻³²P]ATP into histone H₁. Incubation were conducted for 30 min at 30°C in a final volume of 85 ml. In final concentrations, the assay mixture contained 25 mM ATP (0.4 mCi), 10 mM magnesium diacetate, 5 mM β-mercaptoethanol, 50 mg histone H₁, 20 mM HEPES, pH 7.4 and, unless otherwise indicated, 0.2 mM CaCl₂ and 10 mg/ml phosphatidylserine vesicles. The incorporation of³²Pphosphate into histone H₁ was linear for at least 30 min. The reaction was stopped by the addition of 2 ml ice cold 5% TCA, 10 mM H₂PO₄. The radioactivity retained on GPC glass fiber filters after filtration was determined by counting the filters in 2 ml scintillation fluid. PKC activity was determined after subtracting the incorporation in the absence of calcium and phospholipids (31).

**Drugs**

Pilocarpine, pirenzepine, and NCDC were purchased from Sigma-Aldrich. Stock solutions were freshly prepared in the corresponding buffers.

**Statistical analysis**

Student’s t test for unpaired values was used to determine the levels of significance. ANOVA and post hoc test (Dunnett’s method and Student-Newman-Keuls test) were used when pairwise multiple comparison was necessary. Differences between means were considered significant if p < 0.05.

**Results**

**Detection of serum Abs**

To demonstrate the presence of serum IgG directed against cerebral frontal cortex cells in schizophrenic patients, we performed an indirect immunofluorescence assay. Fig. 1A shows a positive stain on the surface of neural cells when they were incubated with IgG from schizophrenic patients. A negative image was obtained when IgG from normal controls were tested (Fig. 1B). These results were assessed using flow cytometry. Fig. 2 shows the binding capacity of schizophrenic patient Abs incubated with neural cells. Whereas control IgG presented low binding (5 ± 0.3%), schizophrenic samples showed a ∼3-fold increase (14 ± 1.2%) in binding to neural cells. The mean fluorescence of control samples was 19 ± 1, whereas that of schizophrenic IgG was 32 ± 2 (p < 0.05).

To test the ability of Abs from schizophrenic patients to interact with cerebral frontal cortex mAChR, immunoblottting, radioligand competition assay, dot blot, and ELISA were performed.

![FIGURE 1. Indirect immunofluorescence staining of cerebral frontal cortex cells. Cell slides were incubated with 1 × 10⁻⁶ M IgG from normal (A) or schizophrenic (B) patients, washed with PBS, and stained with rabbit anti-human IgG FITC-conjugated Fab’₂. The preparation was photographed with a Nikon photomicroscope equipped with epi-illumination. ×450.](http://www.jimmunol.org/)

Downloaded from http://www.jimmunol.org/ by guest on May 1, 2017
Immunoblotting experiments (Fig. 3) showed that affinity-purified anti-M1 peptide IgG from schizophrenic patients revealed a band with a molecular mass coincident with that labeled by an anti-M1 mAChR Ab. Moreover, when the M1 mAChR synthetic peptide (corresponding to second extracellular loop of human M1 mAChR) was included in the reaction or when normal IgG was used, no band was observed.

To thoroughly analyze how the Abs from schizophrenic patients interact with M1 mAChR subtype, a radioligand competition binding assay was performed using [3H]pirenzepine as specific radioligand for M1 mAChR. In saturation studies and Scatchard analysis, an irreversible interaction was established in cerebral frontal cortex membrane exposed to IgG from schizophrenic patients. Thus, Table I shows that cerebral membranes reacted with IgG, decreasing binding sites ($B_{max}$) without changes in equilibrium dissociation constant ($K_d$). On the contrary, normal IgG did not affect $K_d$ or $B_{max}$.

By dot blot and enzyme immunoassays, we determined the molecular interaction between Abs and human M1 mAChR, testing whether the IgG from schizophrenic patients could recognize the human M1 mAChR synthetic peptide. Fig. 4 shows that both sera and IgG from schizophrenic patients, but not those from normal subjects, reacted positively when M1 mAChR peptide was used as the coating Ag. The reaction was neutralized by preincubating the sera or IgG with the corresponding synthetic peptide, pointing to the specificity of the reaction.

By means of ELISAs using the M1 synthetic peptide as Ag, we confirmed the presence of anti-M1 autoantibodies in the sera of schizophrenic patients. Thus, Fig. 5 (top) shows the concentration-dependent increase in OD values with affinity-purified anti-M1 peptide IgG and the corresponding sera from schizophrenic patients, with OD values always >3 SD of those from normal individuals. The specificity of the anti-M1 peptide IgG was assessed by the ability of the M1 synthetic peptide (10-fold concentrated) to inhibit the reaction. Also, Fig. 5 (bottom), shows comparatively the increase in OD values triggered by the anti-M1 peptide IgG and its corresponding sera and total IgG. The concentration of affinity purified anti-M1 peptide IgG ($5 \times 10^{-7}$ M) that maximally increased OD values corresponded to $~5 \times 10^{-6}$ M total IgG concentration. The non anti-M1 peptide fraction eluted from the column showed OD values similar to normal IgG. As expected, the IgG fraction from normal subjects purified by affinity chromatography with the synthetic peptide gave negative results (Fig. 5).

Muscarinic cholinergic receptor-mediated effect of autoantibodies from schizophrenic patients

As already shown, the anti-M1 peptide Ab can react with rat cerebral frontal cortex. Knowing that the amino acid sequence of rat and human M1 synthetic peptide corresponding to the second extracellular loop of M1 mAChR has a strong homology (92%), we studied the muscarinic cholinergic receptor-mediated effect of autoantibodies from schizophrenic patients on rat cerebral frontal cortex. To evaluate the effect of autoantibodies from schizophrenic patients on cerebral intracellular signals coupled to M1 mAChR, changes in cGMP production and inositol phosphates (IPs) accumulation and protein kinase C (PKC) translocation were measured.

As shown in Table II, there was a significant increase in cGMP production by rat cerebral frontal cortex exposed to total IgG or the corresponding affinity purified anti-M1 peptide IgG from schizophrenic patients. These effects resembled those of the authentic

### Table I. Inhibition of [3H]pirenzepine binding on rat cerebral frontal cortex membranes by IgG from schizophrenic patients

<table>
<thead>
<tr>
<th>IgG</th>
<th>$K_d$ (nM)</th>
<th>$B_{max}$ (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.6 ± 0.5</td>
<td>924 ± 59</td>
</tr>
<tr>
<td>Schizophrenic</td>
<td>5.8 ± 0.4</td>
<td>482 ± 33*</td>
</tr>
<tr>
<td>Normal</td>
<td>6.9 ± 0.6</td>
<td>958 ± 43</td>
</tr>
</tbody>
</table>

* Cerebral membranes (0.15–0.20 mg protein) were or were not incubated with $1 \times 10^{-6}$ M IgG from schizophrenic patients or normal subjects in the presence of increasing concentrations of [3H]pirenzepine. Values are calculated from linear regression analysis. Results are means ± SEM of 9 schizophrenic patients and 10 normal subjects performed in duplicate.

* $p < 0.001$ different from normal.
agonist pilocarpine. Furthermore, the maximal increment of cGMP induced by the anti-M₁ peptide IgG could be blunted by pirenzepine (5×10⁻⁶ M) and were neutralized after preincubating the IgG with the synthetic peptide. Normal IgG had no effect on the system studied.

To assess the action of Abs from schizophrenic patients on IP₃ accumulation, slices from rat cerebral frontal cortex were incubated with [myo-³H]inositol which is incorporated into phosphoinositol precursor in the cell, and water-soluble radiolabeled IPs formed in the presence or absence of total IgG or affinity-purified anti-M₁ peptide IgG were determined. Table III shows that total IgG and the corresponding anti-M₁ peptide IgG were able to produce a significant increase in phosphoinositol formation. This stimulatory effect was observed in the second peak with no modification in the first one. The effect of Ab mimicked the pilocarpine action. The anti-M₁ peptide IgG effect could be abolished by preincubating cerebral tissue with pirenzepine. Furthermore, the Ab effect could be blocked by 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate, indicating that phospholipase C-mediated hydrolysis of PIP₂ was involved in this effect. Also, the M₁ peptide neutralized the IgG action, indicating the specificity of the reaction (Table III).

To analyze whether Abs from schizophrenic patients upon brain mAChR lead to PKC activation, rat cerebral frontal cortices were exposed to affinity purified anti-M₁ peptide IgG and PKC enzymatic activity was determined in both cytosolic and membrane fractions. Fig. 6 (left) anti-M₁ peptide IgG induced PKC translocation within 30 min of incubation, increasing the membrane and decreasing the cytosolic activity significantly. The anti-M₁ peptide
IgG effect was blunted by pirenzepine and neutralized by the peptide. At $1 \times 10^{-7}$ M, affinity-purified anti-M$_1$ peptide IgG triggered PKC activity to that of $1 \times 10^{-6}$ M concentrations of the corresponding sera or total IgG, whereas normal IgG showed no effect (Fig. 6, right).

**Discussion**

From a clinical point of view, the course of exacerbations and remissions, genetic vulnerability, and the early onset in many cases hint at parallels between schizophrenia and autoimmune diseases, especially those with overt CNS manifestations (33–35). Also, the symptomatology, i.e., paranoid or negative symptoms, and the acuity stage of the illness influence the immunological parameter (1).

The aim of our study was to examine the possible role of altered humoral immunity exploring the cholinergic muscarinic activity of IgG from chronic schizophrenic patients. In accordance with others (37–41), indirect immunofluorescence assay and flow cytometry provided evidence that certain components of the IgG fraction from these patients can recognize rat cerebral frontal cortex neural cells. The presence of serum Abs was also detected in neurolepticaive (42) and neuroleptic-treated schizophrenic patients (43, 44).

Neurochemical alterations affecting the dopaminergic/cholinergic axis appear to be involved in the pathology of schizophrenia (12, 13). Therefore, in an attempt to elucidate the nature of the parasympathetic mechanism involved, we characterized the participation of the muscarinic cholinergic system in the effect of the Ab. In this sense, we conducted binding, dot blot, ELISA, and Western blot assays.

A concentration-dependent inhibition of specific radioligand to M$_1$ mAChR from rat cerebral frontal cortex was demonstrated. The Ab binds irreversibly to M$_1$, mAChR, decreasing the available binding sites without affecting the receptor affinity. These results are in agreement with those of others (20, 22, 45) that reported a decrease in mAChR density in frontal, parietal, and temporal human schizophrenic brain cortices. This reduced number of mAChR was mainly related to M$_1$ subtype (22). Thus, we encounter the possibility that the lower expression of M$_1$, mAChR in schizophrenic patients is due to Ab fixation to the receptors.

Furthermore, judging by dot blot, ELISA, and immunoblotting results, the Abs reacted molecularly against the second extracellular loop of the human M$_1$ receptor. It has been shown that the second extracellular loop of mAChR appears to be the main immunogenic region of these receptors (46). The specificity of these interactions was assessed by the fact that corresponding affinity-purified antipeptide Ab behaved similar to total IgG.

Schizophrenic patients have several symptoms associated with the parasympathetic system, although the molecular mechanism involved is still unclear. These antipeptide Abs not only were able to interact with the second extracellular loop of the human M$_1$, mAChR but also displayed an agonistic-like activity; they modified the intracellular events associated with specific M$_1$, mAChR activation, i.e., increased cGMP production, activated phosphoinositide turnover, and translocated PKC. All of these biological effects on rat cerebral frontal cortex, triggered by the autoantibodies, were blunted by pirenzepine, neutralized by the peptide, and

**Table II. Changes in cGMP production in rat cerebral frontal cortex by Abs from schizophrenic patients**

<table>
<thead>
<tr>
<th>Additions</th>
<th>cGMP (pmol/mg tissue w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>378 ± 28</td>
</tr>
<tr>
<td>Sch-IgG</td>
<td>1250 ± 42*</td>
</tr>
<tr>
<td>Sch-antipeptide IgG</td>
<td>1120 ± 38*</td>
</tr>
<tr>
<td>Sch-antipeptide IgG + pirenzepine</td>
<td>412 ± 26</td>
</tr>
<tr>
<td>Sch-antipeptide IgG + peptide</td>
<td>392 ± 23</td>
</tr>
<tr>
<td>Peptide</td>
<td>382 ± 30</td>
</tr>
<tr>
<td>Normal IgG</td>
<td>367 ± 27</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>1270 ± 37*</td>
</tr>
</tbody>
</table>

* cGMP was measured after incubating tissue (0.5 g) alone or with $1 \times 10^{-6}$ normal IgG or IgG from schizophrenic patients (Sch-IgG) or $1 \times 10^{-7}$ M affinity-purified anti-M$_1$ peptide IgG (Sch-antipeptide IgG) or pilocarpine for 5 min in the absence or presence of $5 \times 10^{-6}$ M pirenzepine or $1 \times 10^{-5}$ M peptide. The cGMP level was measured in tissue homogenate residues as described in Materials and Methods. Results are mean ± SEM for 10 schizophrenic and normal subjects performed in triplicate.

# Significantly different from normal IgG, $p < 0.001$.

**Table III. Increase of phosphoinositide (PI) turnover in rat cerebral frontal cortex Abs from schizophrenic patients**

<table>
<thead>
<tr>
<th>Additions</th>
<th>PI (area U/mg tissue w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>Sch-IgG</td>
<td>96 ± 5*</td>
</tr>
<tr>
<td>Sch-antipeptide IgG</td>
<td>87 ± 4*</td>
</tr>
<tr>
<td>Sch-antipeptide IgG + pirenzepine</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>Sch-antipeptide IgG + peptide</td>
<td>46 ± 3</td>
</tr>
<tr>
<td>Sch-antipeptide IgG + NCDC</td>
<td>43 ± 4</td>
</tr>
<tr>
<td>Peptide</td>
<td>50 ± 4</td>
</tr>
<tr>
<td>Normal IgG</td>
<td>44 ± 3</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>107 ± 6*</td>
</tr>
</tbody>
</table>

* Rat slices from cerebral frontal cortex were incubated for 30 min with [myo-3H]inositol and for an additional 30 min with or without $5 \times 10^{-6}$ M pirenzepine or $5 \times 10^{-6}$ M NCDC. Tissues were then left for a further 30 min in the absence or presence of $1 \times 10^{-6}$ M normal IgG or IgG from schizophrenic patients (Sch-IgG) or $1 \times 10^{-7}$ M affinity-purified anti-M$_1$ peptide IgG (Sch-antipeptide IgG) or pilocarpine. Results are mean ± SEM for seven schizophrenic and normal subjects performed in triplicate.

# Significantly different from normal IgG, $p < 0.001$. 

**FIGURE 6.** Activation of cerebral frontal cortex PKC by Abs from schizophrenic patients. Left, Time course of activation of PKC by affinity-purified anti-M$_1$ peptide IgG ($1 \times 10^{-7}$ M). PKC activity was measured on cGMP (○) and membrane (●) in the presence of IgG alone (——) or the same IgG preincubated with M$_1$, mAChR synthetic peptide (----). Values were expressed as percentage of total PKC activity. Values are mean ± SEM of 10 schizophrenic patients. Right, Effect of sera (B), total IgG (C), or affinity-purified anti-M$_1$ peptide IgG (D) from schizophrenic patients on both cytosolic (●) and membranes (■) PKC activity on average basal values (A) were analyzed. Cerebral frontal cortex was preincubated before the addition of $1 \times 10^{-7}$ M affinity-purified anti-M$_1$ peptide IgG with $5 \times 10^{-5}$ M pirenzepine (E). Normal IgG was also tested (F). Results are means ± SEM of six different schizophrenic patients and normal individuals performed in duplicate.
resembled the effects of the authentic agonist pilocarpine. Thus, the positive feature of our report is that early agonist-promoting activation in M₁ mAChR initiated by autoantibodies bind to and persistently activate cerebral frontal cholinceptors. Later, the agonistic activity displayed by these autoantibodies could induce desensitization, internalization, and/or intracellular degradation of the mAChR, leading to a progressive decrease of cerebral M₁ mAChR expression and activity. Several lines of research suggested that muscarinic hyperactivity may be implicated in the pathogenesis of negative schizophrenic symptoms (47). In view of the crucial role of the cholinergic system in schizophrenia, Tandon (12) and Carlsson (13) assumed a link between increased dopamine activity and positive symptoms, a direct association of muscarinic activity with negative symptoms, an increase in positive symptoms resulting from decreasing muscarinic activity, evidence of increased muscarinic activity in the psychotic phase, the covariance of positive and negative symptoms in the evolution of the illness, and the presence of several sites of dopamine-cholinergic interactions in the brain. Moreover, Tandon (12) suggested that as dopamine increases at the onset of an acute psychotic exacerbation, cholinergic activity increases as well in the attempt to maintain the dopamine-acetylcholine balance. Studied serum samples were collected during a psychotic exacerbation in chronic paranoid schizophrenic patients. Therefore, it could be hypothesized that the paranoid or negative symptoms and the acuity stage of the illness could influence the muscarinic cholinergic activity by Ab-mAChR-specific interaction. Hence, the autoimmune process could explain the relapsing in the chronic course of this mental disorder (8).

Noy et al. (48) proposed that the following mechanisms may account for an immune etiology in schizophrenia: 1) a viral infection of neural tissue(s) occurs and leads to exposure of brain self-Ag(s), resulting in an autoimmune reaction; and 2) an infection (not necessarily of neural tissue) induces the production of Abs that, as a result of a molecular mimicry, identify brain Ags as non-self and cause an autoimmune reaction. Hence, the disturbance in self-recognition of brain Ags by the immune system is the primary phenomenon, whereas the signs and symptoms of schizophrenia are the secondary manifestations of the immunological attack on the brain. These mechanisms may account for the different stages and/or subtypes of the disease as caused by different brain Ags. It is concluded from our results that there is some evidence for an underlying autoimmune process in schizophrenia. Our results might shed new light on the neurotransmitter hypothesis in mental disorders, possibly leading to new diagnostic and therapeutic regimes.

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References


