A peripheral marker for schizophrenia: Increased levels of D_3 dopamine receptor mRNA in blood lymphocytes

Tal Ilani*, Dorit Ben-Shachar†, Rael D. Strous‡, Marina Mazor§, Ala Sheinkman§, Moshe Kotler‡, and Sara Fuchs*¶

*Department of Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel; †Rambam Medical Center and B. Rappaport Faculty of Medicine, Technion, Haifa 31096, Israel; ‡Beer Yaacov Mental Health Center, Beer Yaacov 70300, Israel; and §Mental Health Center, Tirat Hacarmel, Haifa 30200, Israel

Communicated by Pedro M. Cuatrecasas, University of California, San Diego School of Medicine, Rancho Santa Fe, CA, November 9, 2000 (received for review September 26, 2000)

Dopamine is a major neurotransmitter in the central nervous system, and its receptors are associated with a number of neuropathological disorders such as Parkinson's disease and schizophrenia. Although the precise pathophysiology of schizophrenia remains unknown, the dopaminergic hypothesis of the illness assumes that the illness results from excessive activity at dopamine synapses in the brain. Because, at present, the diagnosis of schizophrenia relies on descriptive behavioral and symptomatic information, a peripheral measurable marker may enable a simpler, more rapid, and more accurate diagnosis and monitoring. In recent years, human peripheral blood lymphocytes have been found to express several dopamine receptors (D_3, D_4, and D_5) by using molecular biology techniques and binding assays. It has been suggested that these dopamine receptors found on lymphocytes may reflect receptors found in the brain. Here we demonstrate a correlation between the D_3 dopamine receptor on lymphocytes and schizophrenia and show a significant elevation of at least 2-fold in the mRNA level of the D_3, but not of the D_4, dopamine receptor in schizophrenic patients. This increase is not affected by different antipsychotic drug treatments (typical or atypical). Moreover, nonmedicated patients exhibit the same pattern, indicating that this change is not a result of medical treatment. We propose the D_3 receptor mRNA on blood lymphocytes as a marker for identification and followup of schizophrenia.

Schizophrenia is a neuropsychiatric disorder afflicting about one percent of the population. Although its exact pathogenesis is still not known precisely, a common belief is that excessive activity at dopaminergic synapses in the brain plays a prominent role (1). To date, a definitive diagnosis of schizophrenia requires a 6-month duration of symptomatology and relies on heterogeneous symptoms. Because there is neither an effective biological marker for identifying schizophrenia (2, 3), nor an accurate and rapid diagnosis to ensure more optimal management at an early stage in the illness (4), there remains a vital need for a convenient assay for diagnosis and followup of schizophrenia.

Most of the drugs used to treat schizophrenia act to control symptoms by neuroreceptor antagonism. Moreover, the dopaminergic basis of schizophrenia is strongly supported by the close correlation between clinical efficacy of antipsychotic medications and their potency to antagonize the binding of dopamine to its receptors (5).

Dopamine receptors are divided into two subclasses, D1 and D2. The D1 subclass contains the D_1 and D_5 receptor subtypes, and the D2 subclass contains the D_2, D_3, and D_4 subtypes (6). The dopamine hypothesis of schizophrenia specifically relates to the D2 subclass. Notably, most drugs effective in treating schizophrenia exhibit D2 receptor antagonistic activity, and administration of a selective D1-like antagonist has been reported to result in the worsening of symptoms (7). The D_3 receptor, one among the three in the D2 subclass, is located principally in an area of the brain that could be very relevant to schizophrenia, the nucleus accumbens (2). Studies with positron-emission tomography and postmortem brain tissue have indicated increased levels of D2-like dopamine receptors in schizophrenic patients when compared with nonschizophrenic patients (8). Thus, the level of dopamine receptor could be used as a marker for schizophrenia if it could be analyzed on an available tissue, preferably a peripheral one.

In recent years, high-affinity binding of dopaminergic ligands, as well as the presence of mRNA of several dopamine receptor subtypes (D_3, D_4, and D_5) in human peripheral blood lymphocytes (PBLs), have been reported (9, 10). It should be noted, however, that neither D_2 nor D_1 dopamine receptor subtypes, which are the most abundant receptors in the brain and belong to the D2 and the D1 subclasses, respectively, have been detected in lymphocytes. Although the significance of dopamine receptors, as well as of other neurotransmitter receptors, in lymphocytes is still not clear, it has been suggested that they may reflect corresponding brain receptors. Several studies have demonstrated the increased binding of dopamine antagonists in lymphocytes of schizophrenic patients as compared with healthy individuals (11, 12). In addition, a previous study carried out in our lab has demonstrated that spiperone (a D2 antagonist) binding in peripheral blood lymphocytes is higher in neuroleptic responders as compared with treatment-resistant schizophrenic patients (13). However, the observed differences in binding studies were rather low and often not significant. The discrepancies obtained could have resulted from the crossreactivity of radioligands with different subtypes of the receptor and with other receptors (e.g., serotonergic), and from the scattered levels of binding sites. Therefore, such binding assays in lymphocytes may not be suitable as reliable assays for schizophrenia.

In this work, we have measured mRNA levels of dopamine receptors in the PBLs of schizophrenic patients and healthy individuals to find out whether the receptors can serve as peripheral markers for this disorder. Because the inhibitory D2 subclass, rather than the D1 subclass, of dopamine receptors is considered to be associated with neuropsychiatric disorders, in this study we have focused on only the D_3 and D_4 subtypes, both belonging to the D2 subclass. In the current study, we demonstrate a correlation between the D_3 dopamine receptor on lymphocytes and schizophrenia and show a significant elevation (2- to 7-fold) in the mRNA level of D_3, but not of D_4, in schizophrenic patients. This increase is not affected by different antipsychotic drug treatments (typical or atypical). Moreover,

Abbreviation: PBL, peripheral blood lymphocyte.

¶To whom reprint requests should be addressed. E-mail: sara.fuchs@weizmann.ac.il.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.021535398. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.021535398

MEDICAL SCIENCES

Table 1. Characterization of patients analyzed in this study

Number	Age	Sex	Diagnosis	Comments
Schizophrenic patients				
S1	21	M	Schizophrenia–residual type	
S2	27	M	Schizophrenia–paranoid type	
S3	25	M	Schizophrenia–undifferentiated type	
S4	27	F	Schizophrenia–unspecified type	
S5	49	F	Schizophrenia–paranoid type	
S6	57	F	Schizophrenia–residual type	
S7	41	M	Schizophrenia–undifferentiated type	
S8	54	M	Schizophrenia–paranoid type	
S9	47	M	Schizophrenia–undifferentiated type	
S10	42	M	Schizophrenia–undifferentiated type	
S11	42	M	Schizophrenia–paranoid type	
S12	21	M	Schizophrenia–undifferentiated type	Nonmedicated
S13	40	F	Schizophrenia–paranoid type	Nonmedicated
S14	29	M	Schizophrenia–paranoid type	Nonmedicated
Healthy controls				
C1	45	F	—	—
C2	37	F	—	—
C3	37	M	—	—
C4	62	F	—	—
C5	22	M	—	—
C6	44	M	—	—
C7	31	M	—	—
C8	32	M	—	—
C9	49	F	—	—
C10	27	M	—	—
C11	36	F	—	—

nonmedicated patients exhibit the same pattern, indicating that this change is not a result of medical treatment. We propose the D_3 receptor mRNA as a peripheral marker for identification and followup of schizophrenia.

Materials and Methods

Patients. Schizophrenic patients were recruited from Tyrat Hacarmel and Beer Yaacov Mental Health Centers after providing written informed consent for participation in the study. The study has been approved by the Institutional Review Boards for human studies in these two mental health centers. All patients were diagnosed formally according to the *Diagnostic and Statistical Manual of Mental Disorders*, Fourth Edition (DSM-IV; ref. 14) and were evaluated by using standard rating scales by a senior psychiatrist. Healthy individuals' ages and sexes matched the patient group as much as possible.

Lymphocyte Isolation. Blood (40–50 ml) was drawn from the cubital vein into a heparinized plastic syringe and then transferred into a sterile 50-ml plastic tube. Blood samples were diluted with an equal volume of PBS, were placed onto Ficoll/Paque gradients, and then were centrifuged for 30 min at 400 × *g*. The lymphocyte layer was collected and washed twice in PBS. The resulting pellet was frozen immediately at −80°C until RNA preparation.

Reverse Transcription–PCR Analysis. Total RNA was isolated from lymphocytes by using the guanidinum-thiocyanate method, and the amount and quality of RNA were determined by using spectrophotometry and gel electrophoresis (2% agarose; GIBCO/BRL). Two micrograms of total RNA was reverse transcribed into first-strand cDNA by using polydT-priming and 20 units of Moloney murine leukemia virus reverse transcriptase. Two microliters cDNA product (80 ng RNA) was used for PCR amplification at a final concentration of 1× PCR buffer (Perkin–Elmer) and 1 unit of *Taq* DNA polymerase (Perkin–Elmer), in a final volume of 25 μl. PCR was carried out in a DNA thermocycler (MiniCycler; MJ Research, Cambridge, MA) for

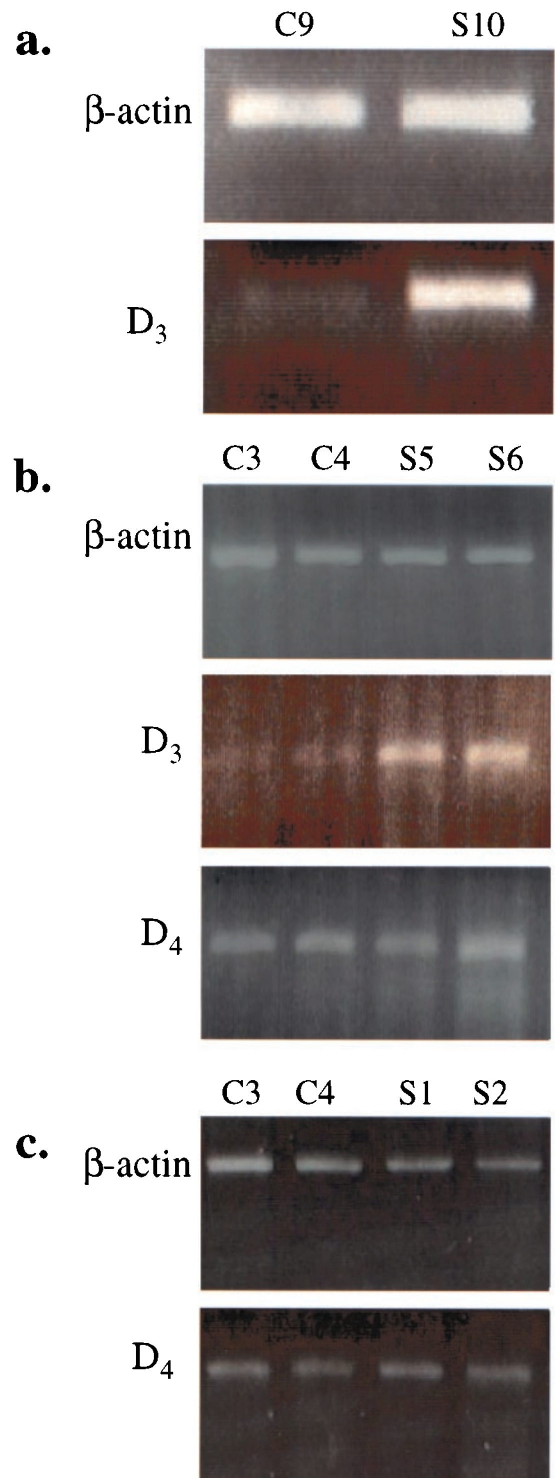

Fig. 1. Ethidium bromide staining of D_3, D_4, and β-actin PCR products from healthy controls (C) and schizophrenics (S).

Table 2. Densitometric evaluation of D_3 and D_4 mRNA levels in patients compared with their levels in healthy individuals

Schizophrenic patients				Controls					Schizophrenic patients			Controls				
Number	β-actin arb. units	D_3 arb. units	D_3/β-actin	Number	β-actin arb. units	D_3 arb. units	D_3/β-actin	D_3 fold increase, S/C	β-actin arb. units	D_4 arb. units	D_4/β-actin	Number	β-actin arb. units	D_4 arb. units	D_4/β-actin	D_4 fold increase, S/C
S1	67	51	0.671	C3	86	23	0.267	2.513	74	70	0.945	C3	67	70	1.044	0.905
S2	87	56	0.643	C3	86	23	0.267	2.408	71	72	1.010	C3	67	70	1.044	0.967
S3	82	60	0.731	C3	86	23	0.267	2.737	72	74	1.027	C3	67	70	1.044	0.983
S4	98	100	1.020	C2	95	61	0.642	1.588	67	71	1.059	C2	82	73	0.890	1.189
S5	55	143	2.600	C2	58	59	1.017	2.556								
S6	75	153	2.040	C4	79	70	0.886	2.302								
S7	19	171	9.000	C10	85	163	1.917	4.694								
S8	138	426	3.087	C8	425	176	0.414	7.456								
S9	89	60	0.674	C7	121	28	0.231	2.917								
S10	107	71	0.663	C7	121	28	0.231	2.870								
S11	303	216	0.714	C3	271	29	0.107	6.644								
S12	319	227	0.711	C7	273	86	0.315	2.257								
S13	237	354	1.493	C9	199	130	0.653	2.286								
S14	162	825	5.092	C8	91	60	0.659	7.727								

arb., arbitrary.

23 cycles (β-actin) and 38 cycles (D_3 and D_4) with an annealing temperature of 60°C. The amplification was found to be linear between 30 and 40 cycles for D_3 and D_4, and between 19 and 25 cycles for β-actin.

The PCR primers for D_3, D_4, and β-actin were designed to include at least one intron to eliminate amplification of genomic DNA. Their sequences were as follows:

D_3 dopamine receptor—GGAGACGGAAAAGGATCCT-CACTCG (nucleotides 655–680) and TCAGCAAGACAG-GATCTTGAGGAAGG (nucleotides 1203–1177).

D_4 dopamine receptor—CGGGATCCCACCCCAGACTC-CACC (nucleotides 964–988) and CGGAATTCCGTTGCG-GAACTCGGC (nucleotides 1240–1216).

β-actin—TGAAGTGTGACGTGGACATCCG (nucleotides 96–117) and GCTGTCACCTTCACCGTT CCAG (nucleotides 543–522).

The obtained PCR products corresponded to the respective dopamine receptor fragments, as confirmed by sequence and Southern blot analyses.

Quantification of PCR products was performed by using a densitometer and SCION IMAGE (Frederick, MD) analysis software, and/or PCR–ELISA.

PCR–ELISA. PCR was performed as described previously except for the use of digoxigenin-labeled dNTPs. PCR products were incubated with biotinylated-specific internal primers of the tested fragments that were immobilized in streptavidin-coated microtiter plates. The biotinylated internal primers served as capture probes. The bound digoxigenin-labeled PCR products then were incubated with anti-digoxigenin-peroxidase conjugate that bound to the digoxigenin residues in the labeled PCR product. Peroxidase substrate solution was added, and the color developed was measured in a microtiter-plate reader.

Results and Discussion

Table 1 summarizes the details (ages, sexes, and diagnoses) of schizophrenic patients and healthy controls from whom blood samples were obtained for this study. RT-PCR was performed on total RNA preparations from these blood samples with primers specific for D_3 or D_4 dopamine receptor and β-actin as a control. The specific PCR products were resolved on 2% agarose gels, and their sequences were verified. For each patient, a sex- and optimal-age-matched healthy control was used, and the level of specific dopamine receptor mRNAs was compared between sick and healthy patients. As depicted in Fig. 1 *a* and *b* for several representative patients, the signals for D_3 receptor mRNA were significantly higher in schizophrenic patients than in healthy controls. This increase was found to apply to the D_3 receptors specifically, because no significant differences in the intensities of D_4 receptor bands were detected between schizophrenic patients and healthy controls (Fig. 1 *b* and *c*).

Quantification of the intensities of the specific D_3 receptor bands was performed by densitometry. The results for 14 patients are summarized in Table 2. Each schizophrenic patient is compared with a sex- and optimal-age-matched healthy individual. For each of them, a ratio of the measured density value for the D_3 receptor to the value for β-actin was determined. The ratio of these two values for a patient and a matched healthy

Table 3. Evaluation by PCR–ELISA of D_3 mRNA levels in patients compared with their levels in healthy individuals

Schizophrenic patients				Controls				
Number	β-actin, OD	D_3, OD	D_3/β-actin	Number	β-actin, OD	D_3, OD	D_3/β-actin	D_3 fold increase, S/C
S1	0.556	0.868	1.561	C8	0.918	0.552	0.601	2.597
S2	0.808	2.225	2.753	C9	0.405	0.330	0.814	3.382
S3	0.224	0.253	1.129	C8	0.533	0.272	0.510	2.214
S4	0.629	0.394	0.626	C8	0.876	0.316	0.360	1.738
S5	0.340	0.823	2.420	C2	0.365	0.533	1.460	1.657
S6	0.339	0.899	2.652	C3	0.368	0.444	1.206	2.199

OD, optical density at 405 nm.

control, respectively, represents the increased level (in folds) in D_3-specific mRNA in a given patient. As shown in Table 2, the increased levels obtained for the 14 patients ranged from 1.59 to 7.73 (average increase 3.64 ± 2.09). This increase in D_3 receptor mRNA in schizophrenic patients is significantly higher than the reported increases in binding levels and other recently suggested peripheral markers for schizophrenia (15). Furthermore, the increase in D_3 receptor RNA was unaffected by different drug treatments. Although some of the patients received typical treatment and some atypical treatment (see Table 1), it can be noted that all patients exhibited a similar range of increase indicating that this was not a result of specific dopamine-receptor subtype blockade and up-regulation. Moreover, we found that this increase was not the consequence of dopamine-receptor antagonist treatment, because nonmedicated patients (S12, S13, and S14) showed a similar increase in D_3 level (see Tables 1 and 2).

Another way to quantify the differences in a specific mRNA level was obtained from PCR–ELISA experiments (see *Materials and Methods*). Table 3 summarizes the results obtained from 6 patients. The increased mRNA levels observed are between 1.66 and 3.38 (average increase 2.30 ± 0.63). It should be noted that there is a relatively good agreement between the quantitative values obtained by densitometry or PCR–ELISA (see patients S1, S4, and S6 in Tables 2 and 3).

It should be added that the use of sex- and/or age-matched controls does not appear to be critical. We have demonstrated that the differences in D_3-specific mRNA levels between schizophrenic patients and healthy individuals, determined by either densitometry or PCR–ELISA, were similar when compared with additional, not necessarily matched, controls (Table 4). This observation may be valuable in designing a practical assay.

As mentioned above, the elevated levels of D_3 dopamine receptor in PBLs of schizophrenic patients are in agreement with other reports (7) demonstrating elevated levels of this receptor in postmortem brains of medicated and nonmedicated schizophrenic patients. Such a correlation between the status of receptors in the brain and in PBLs has also been demonstrated in Alzheimer's disease, where muscarinic receptors are reduced in both brains and lymphocytes (16). A previous study by Nagai *et al.* (17) demonstrates that patients with Parkinson's disease exhibit reduced levels of D_3-receptor mRNA in PBLs, as compared with healthy individuals. These latter findings provide another example of a disease that is associated with an insult in the central nervous system that is reflected in PBLs. This reduction has also been detected in medicated and nonmedicated patients. The changes in mRNA levels observed in this study might reflect a systemic imbalance in D_3-receptor level or in brain-receptor status. Further studies are required to determine whether the levels of D_3 receptor correlate with the progression of the disease and whether they can be deployed to monitor changes in the patient's condition.

Table 4. Evaluation of D_3 mRNA levels in patients compared with their levels in healthy individuals

Schizophrenic patients		Controls		
Number	D_3/β-actin	Number	D_3/β-actin	Ratio
S8	3.087	C8	0.414	7.456
		C9	0.498	6.198
S5	2.600	C2	1.017	2.556
		C3	1.145	2.270
		C4	0.886	2.934
S6	2.040	C2	1.017	2.005
		C3	1.145	1.781
		C4	0.886	2.302
S4	1.020	C2	0.642	1.588
		C8	0.656	1.554

In conclusion, our findings strongly suggest that D_3-receptor mRNA levels in PBLs may function as convenient and reliable peripheral markers for schizophrenia and thus assist in the early diagnosis (which is frequently unclear, e.g., ref. 4) and possible followup of the illness. Early diagnosis and treatment of schizophrenia may have prognostic significance, because many consider that more optimal management at an early stage of the illness may alter its course (18). In this manner, our observations would be of significant clinical and practical relevance. Although our findings are certainly robust, we still do not know whether differences in the degrees of increase of specific D_3-receptor mRNA reflects, in any way, the severity and prognosis of the disease. In addition to serving potentially as a peripheral marker, these changes in the D_3-receptor subtype may further indicate its involvement centrally in the pathophysiology of schizophrenia and thus may potentially play a role in the development of medication suitable for management of the chronic disorder. Further studies, clearly warranted to test these observations, are now under way.

We thank Dr. Lily Raveh for encouragement, help, and fruitful discussions, and Shari Carmon for technical assistance. This research was supported in part by a research grant from the Abramson Family Foundation (to S.F.).

1. Carlsson, A., Waters, N. & Carlsson, M. L. (1999) *Eur. Arch. Psychiatry Clin. Neurosci.* **249,** 37–43.
2. Willner, P. (1997) *Int. Clin. Psychopharmacol.* **12,** 297–308.
3. Hietala, J. & Syvalahti, E. (1996) *Annu. Rev. Med.* **28,** 557–561.
4. Sheitman, B. B., Lee, H., Strauss, R. & Lieberman, J. A. (1997) *Schizophr. Bull.* **23,** 653–661.
5. Creese, I., Burt, D. R. & Snyder, S. H. (1976) *Science* **192,** 481–483.
6. Levant, B. (1997) *Pharmacol. Rev.* **49,** 231–252.
7. Karlsson, P., Smith, L., Farde, L., Harnryd, C., Sedvall, G. & Wiesel, F. A. (1995) *Psychopharmacology* **121,** 309–316.
8. Seeman, P. & Niznik, H. B. (1990) *FASEB J.* **4,** 2737–2744.
9. Ricci, A., Bronzetti, E., Felici, L., Tayebati, S. K. & Amenta, F. (1997) *Neurosci. Lett.* **229,** 130–134.
10. Takahashi, N., Nagai, Y., Ueno, S., Saeki, Y. & Yanagihara, T. (1992) *FEBS Lett.* **314,** 23–25.
11. Bondy, B., Ackenheil, M., Birzle, W., Elbers, R. & Frohler, M. (1984) *Biol. Psychiatry* **19,** 1377–1393.
12. Bondy, B., Ackenheil, M., Elbers, R. & Frohler, M. (1985) *Psychiatry Res.* **15,** 41–48.
13. Grodzicki, J., Pardo, M., Schved, G., Schlosberg, A., Fuchs, S. & Kanety, H. (1990) *Biol. Psychiatry* **27,** 1327–1330.
14. American Psychiatric Association (1994) *Diagnostic and Statistical Manual of Mental Disorders* (Am. Psychiatric Assoc., Washington, DC), 4th Ed.
15. Avissar, S., Nechamkin, Y., Barki-Harrington, L., Roitman, G. & Schreiber, G. (1997) *J. Affect Disord.* **43,** 85–93.
16. Ferrero, P., Rocca, P., Eva, C., Benna, P., Rebaudengo, N., Ravizza, L., Genazzani, E. & Bergamasco, B. (1991) *Brain* **114,** 1759–1770.
17. Nagai, Y., Ueno, S., Saeki, Y., Soga, F., Hirano, M. & Yanagihara, T. (1996) *Neurology* **46,** 791–795.
18. Lieberman, J. A. (1999) *J. Clin. Psychiatry* **60,** 9–12.