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The Modulation of the Human Sodium Iodide Symporter Activity by Graves' Disease Sera*

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ABSTRACT

The transport of iodide into the thyroid, catalyzed by the Na^+/I^- symporter (NIS), is the initial and rate-limiting step in the formation of thyroid hormones. To study the basic characteristics of the human (h) NIS, we have established a Chinese hamster ovary cell line stably expressing the hNIS (CHO-NIS9). In agreement with previous work on the rat NIS, iodide uptake in these cells was initiated within 2 min of the addition of ^{131}I, reaching a plateau after 30 min. Both perchlorate and thiocyanate inhibited iodide uptake in a dose-dependent manner, with inhibition evident at concentrations of 0.01 and 0.1 μmol/L, respectively, and reaching complete inhibition at 20 μmol/L and 500 μmol/L, respectively. Ouabain, which blocks the activity of the Na^+/K^+ adenosine triphosphatase, also inhibited iodide uptake in a dose-dependent manner, starting at concentrations of 100 μmol/L and reaching maximum inhibition at 1600 μmol/L, indicating that iodide uptake in these cells is sodium dependent.

CHO-NIS9 cells were further used to study 88 sera from patients with Graves' disease, for iodide uptake inhibitory activity, which were compared with sera from 31 controls. Significant iodide uptake inhibition was taken as any inhibition in excess of the mean + 3 SD of the results with the control sera. On this basis, 27 (30.7%) of the Graves' sera, but none of the controls, inhibited iodide uptake in CHO-NIS9. IgGs from these patients also inhibited iodide uptake, indicating that this inhibitory activity was antibody mediated.

In summary, we have established a CHO cell line stably expressing the hNIS and shown that antibodies in GD sera can inhibit iodide uptake in these cells. This further emphasizes the role of NIS as a novel autoantigen in thyroid immunity. (*J Clin Endocrinol Metab* **83:** 1217–1221, 1998)

IODIDE concentration by the thyroid gland, mediated by the sodium iodide symporter (NIS), is a key step in the formation of iodine-containing thyroid hormones (1, 2).

Several pieces of evidence indicate that the NIS is a candidate autoantigen in autoimmune thyroid disease (ATD) (3). Serum from a single patient with Hashimoto's thyroiditis (HT) inhibited iodide uptake in dog thyrocyte cultures, and similar effects were obtained using monoclonal antibodies raised in mice against the thyroid cell membranes (4). With the cloning and sequencing of the rat (r) NIS (5), IgGs from patients with Graves' disease (GD), and to a lesser extent HT, have been shown to bind to rNIS peptides in an enzyme-linked immunosorbent assay system, most notably peptides corresponding to the 8th and the 12th extracellular domains (6). Western blotting has shown that sera from 4 patients with HT, out of 34 studied, reacted with recombinant rNIS and with a synthetic peptide corresponding to the 6th extracellular loop of the molecule (7, 8). Furthermore, IgGs prepared from these sera inhibited iodide uptake in a Chinese hamster ovary cell line stably expressing the rNIS (8, 9). GD sera showed even higher reactivity to recombinant rNIS using Western blotting (7), but iodide uptake inhibitory activity of these sera was not investigated.

These above findings indicate the presence of antibodies against the NIS in ATD patient sera, in particular those with GD. However, all of these studies have focused only on the rNIS. To confirm that antibodies also react with human (h) NIS, we have established a CHO cell line stably expressing the hNIS (CHO-NIS9) and characterized its basic features. We have subsequently analyzed the effects of GD sera on the human symporter activity using the CHO-NIS9 cell line.

Materials and Methods

Patient sera and IgG preparation

A total of 88 sera from patients with GD were obtained after informed consent and tested for iodide uptake inhibitory activity. Diagnosis was based on the presence of hyperthyroidism and a diffuse goiter, supported by one or more of the following features: the presence of thyroglobulin and/or thyroid peroxidase antibodies, ophthalmopathy, and a personal or family history of organ-specific autoimmunity. Of the 88 patients studied, 26 were newly diagnosed untreated GD patients, and 25 had associated ophthalmopathy (grade II-IV) of whom 9 had also pretibial myxedema (PTM). Sera were also obtained from 31 healthy controls (24 blood donors and 7 normal laboratory volunteers) and from 31 patients with multinodular goiter disease (19 euthyroid and 12 thyrotoxic). Sera were heat inactivated at 56 C° for 60 min. Serum from 6 patients and 6 controls was run through a protein G-Sepharose column (Pharmacia, Milton Keynes, UK) with 10 mmol/L phosphate buffer at pH 7.0. The bound IgG was eluted with 100 mmol/L glycine-HCl at pH 2.8 and was extensively dialyzed against PBS.

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Establishment of CHO cell line expressing the hNIS

The hNIS complementary DNA (cDNA) cloned into the eukaryotic expression vector pcDNA3 and encoding amino acid 1 to 612 of hNIS was a gift from Dr. S. M. Jhiang (The Ohio State University, Columbus, Ohio) (10). This was introduced into CHO-K1 cells by lipofection using Transfectam (Promega, Southampton, UK), according to the manufacturer's protocol. Briefly, CHO-K1 cells were plated out in 90-mm dishes. When cells reached 60% confluence, they were incubated with 11 μg hNIS-pcDNA3 vector and 26.8 μL transfectam reagent in 4 mL serum-free Ham's F-12 medium (Gibco, Paisley, UK). After 6 h incubation, fresh medium containing 10% FCS was added. Twenty-four hours later, selection was started with 400 μg/mL Geneticin (Gibco), which was further increased to 800 μg/mL after 48 h. Surviving colonies (n = 24) were picked up by filter paper discs and were subjected to screening with ^{131}I. One cell line accumulated the highest levels of ^{131}I, termed CHO-NIS9, and was used for further studies. Two other cell lines also accumulated high levels of ^{131}I, approaching 90–95% of CHO-NIS9.

The detection of the symporter gene in CHO-NIS9 cells

RNA was extracted from CHO-NIS9 using TRIzol, according to the manufacturer's protocol (Gibco). RT-PCR was subsequently carried out using hNIS-specific primers, as previously described (11). To provide a positive control, primary thyroid cells were cultured from a patient with GD, as described elsewhere (12), and RNA was extracted from these cells, as above. Amplifications were carried out using cycles of 94 C for 1 min, 55 C for 1 min, and 72 C for 1 min for 30 cycles. PCR was also performed on RNA not subjected to reverse transcription to ensure that the signal detected was not caused by contamination by plasmid containing the hNIS.

The oligonucleotide primers of NIS were designed according to published sequences (10). The sequences were as follows: Sense hNIS: 5′ CTC CCT GCT AAC GAC TCC AG 3′; Antisense hNIS: 5′ AAC AGA CGA TCC TCA TTG GTG 3′.

Iodide uptake

CHO-NIS9 cells were cultured in 6-well plates, and iodide uptake was tested when these cells reached 100% confluence. ^{131}I, at 1.5–2 kBq/mL, was incubated with the cells in serum-free F-12 medium for the period required. Cells were then washed quickly with PBS and solubilized with 1 mL of 1 mol/L sodium hydroxide, and radioactivity was counted using a γ counter.

To determine the effects of GD sera on the symporter activity, 100 μL serum in 1 mL medium (or 0.5–5 mg/mL IgG) was incubated with CHO-NIS9 cells at 37 C for 60 min. An additional 1 mL medium containing 3–4 kBq of ^{131}I was added, and cells were incubated for a further 30 min, followed by washing and solubilization as above.

All experiments were performed in triplicate cultures, except for the inhibition studies, which were done in duplicate cultures. All positive results from the inhibition experiments were repeated at least once to confirm the findings. Results are expressed as percentage of inhibition of iodide uptake, with maximum uptake routinely between 400–700 cpm.

Iodide efflux

Iodide efflux was performed as described by Kosugi *et al.* (9), except that complete medium was used for incubation instead of HBSS buffer.

Statistical analysis

Differences in iodide uptake inhibitory activity of GD and control sera were analyzed using the Mann-Whitney test, and Student's *t* test was applied to test significant differences in iodide uptake inhibitory activity of GD and control IgGs. GD patients were further divided into three subgroups: untreated patients, those with thyroid associated ophthalmopathy (TAO), and patients with PTM. Differences in the frequency of iodide uptake inhibitory activity of patient sera from these subgroups were tested using 2×2 contingency tables. Correlation was analyzed using Pearson's correlation test.

Results

Confirmation of hNIS transfection into CHO-K1 cells

Detection of hNIS messenger RNA in CHO-NIS9 cells was carried out using RT-PCR (Fig. 1a). hNIS-specific PCR product was detected in CHO-NIS9 cells, as well as the positive control prepared from the primary thyroid cell culture. No signal was detected from CHO-K1 cells or RNA prepared from CHO-NIS9 without reverse transcription, ruling out the possibility of contamination with vector containing the hNIS. The presence of functional symporter was further confirmed by iodide uptake studies on both the transfected and the wild-type CHO-K1 cells (Fig. 1b). Iodide uptake in transfected CHO-NIS9 increased 10-fold above background and was completely abolished by incubating the cells with 100 μmol/L perchlorate. In contrast, iodide uptake in wild-type CHO-K1 cells did not increase above background.

Time course of iodide uptake

We examined iodide uptake in CHO-hNIS 2, 5, 6, 10, 15, 30, and 60 min after the addition of ^{131}I. Iodide uptake reached maximal levels (100%) after 30 min, with half-maximal levels (50%) observed after 10 min (Fig. 2). This experiment was repeated three times with similar findings.

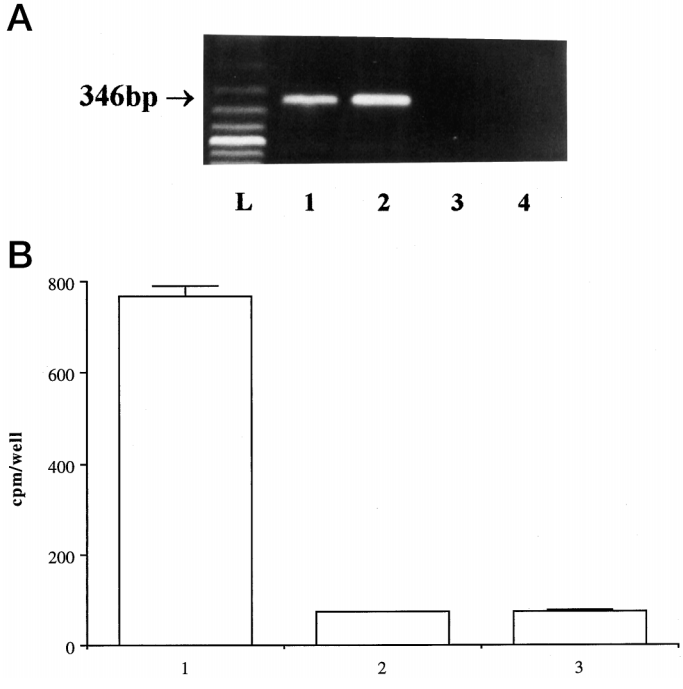

FIG. 1. Confirmation of hNIS expression in CHO-NIS9 cell line. A, Amplification of hNIS-specific PCR product from the cDNA of primary thyroid cells (lane 1), CHO-NIS9 cell line (lane 2), untransfected CHO-K1 cells (lane 3), and from RNA of CHO-NIS9 cells without reverse transcription (lane 4). L represents 100-bp DNA ladder; B, Iodide uptake of CHO-NIS9 cultured in 6-well plates, 30 min after the addition of ^{131}I (column 1), CHO-NIS9 in the presence of 100 μmol/L sodium perchlorate (column 2), and untransfected CHO-K1 cells (column 3). Results are the mean of triplicate cultures ± SEM and are presented as counts per min (cpm)/well.

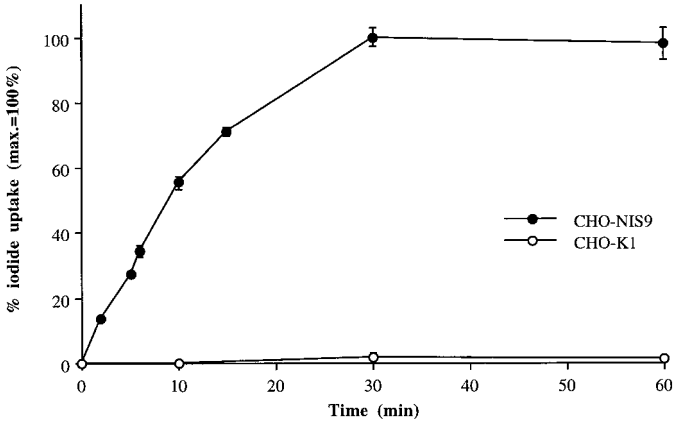

FIG. 2. Time course of iodide uptake in CHO-NIS9 and CHO-K1 cells after addition of ^{131}I. Results are the mean of triplicate cultures ± SEM and are presented as a percentage of the maximal levels of iodide uptake, reached after 30 min.

The effects of perchlorate, thiocyanate, and ouabain

Both perchlorate and thiocyanate inhibited iodide uptake in CHO-NIS9 in a dose-dependent manner, starting from concentrations as low as 0.01 and 0.1 μmol/L, respectively, and reaching complete inhibition with concentrations of 20 and 500 μmol/L, respectively (Fig. 3, a and b). Ouabain also inhibited iodide uptake in a dose-dependent manner, starting from concentrations of 100 μmol/L and reaching a peak (up to 85% inhibition) with 1600 μmol/L (Fig. 3c). These experiments were performed at least twice, with similar results.

Iodide efflux

Iodide efflux properties of CHO-NIS9 were similar to (but slightly faster than) those reported for the rNIS. At 10 and 25 min, 50% and 80% of ^{131}I was released from CHO-NIS9 (Fig. 4), compared with 35 and 70%, respectively, for the rNIS (9).

Inhibition of iodide uptake by GD sera

Thirty-one normal sera were tested for iodide uptake inhibitory activity, with a mean inhibition of less than 17%. Any GD serum with an inhibition of more than 30% (mean of controls + 3 SD) was regarded as positive. A total of 88 GD sera were tested, of which 27 were inhibitory (30.7%). Patients with associated ophthalmopathy, but without PTM, had the highest frequency of NIS inhibitory antibodies (6 of 14; 42.9%), but this difference was not statistically significant ($P > 0.05$) (Fig. 5, Table 1). Sera from patients with multinodular goiter (n = 31; 12 thyrotoxic) also were analyzed, of which 2 (6.5%; 1 thyrotoxic and 1 euthyroid) inhibited iodide uptake.

Purified IgGs (2 mg/mL) from six GD patients, selected for study because the corresponding sera inhibited ^{131}I uptake and because sufficient serum was available for study, also inhibited iodide uptake, by 7–44%, compared with normal controls, which did not inhibit (Fig. 6). All GD IgGs inhibited iodide uptake in a dose-dependent manner, and the effects of different concentrations of IgGs (0.5, 1.25, 2.5, 3.75, and 5 mg/mL) from two GD patients and one normal control are shown in Fig. 7. There was no inhibitory effect of GD IgG

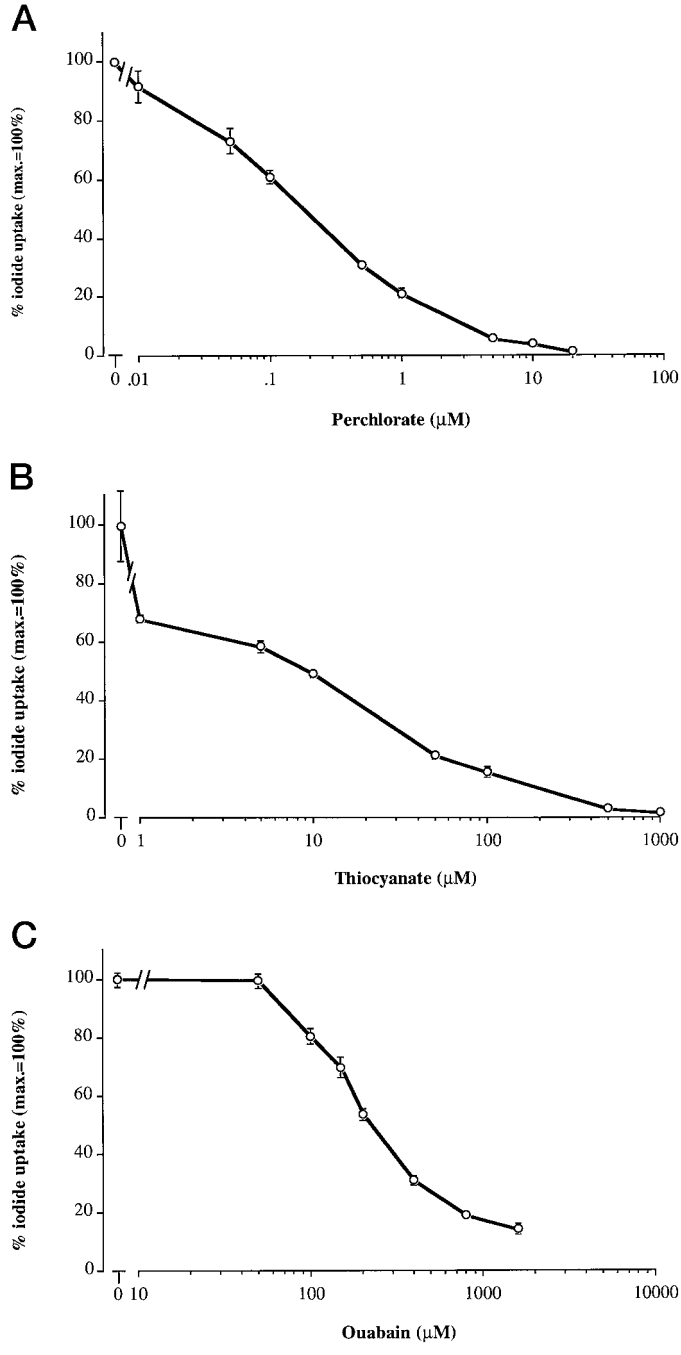

FIG. 3. The effects of perchlorate (Fig. 3A), thiocyanate (Fig. 3B), and ouabain (Fig. 3C) on iodide uptake by CHO-NIS9 30 min after ^{131}I addition. Results are the mean of triplicate cultures ± SEM and are presented as a percentage of the maximal levels of iodide uptake.

preparations at concentrations below 0.1 mg/mL (data not shown). These experiments were repeated at least twice, with similar results.

Comparing control and GD sera, a significant difference in iodide uptake inhibitory activity was detected between the two groups ($P = 0.003$). IgGs from these two groups also showed a difference in iodide uptake inhibitory activity ($P = 0.005$), indicating that the effects of the patient sera are mediated by an antibody. There was also a dif-

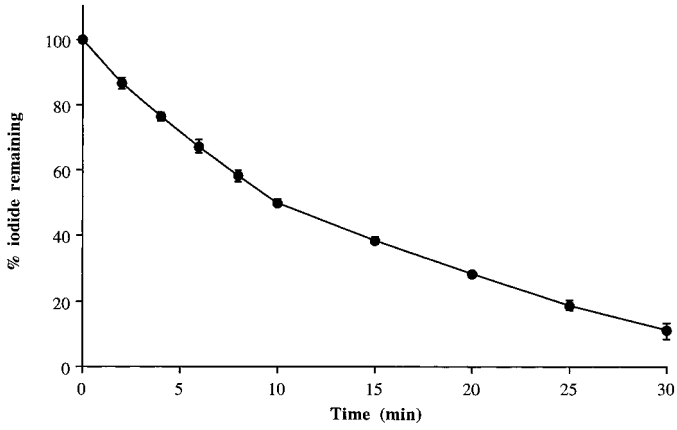

FIG. 4. Iodide efflux from CHO-NIS9. Results are the mean of triplicate cultures ± SEM (% remaining of ^{131}I).

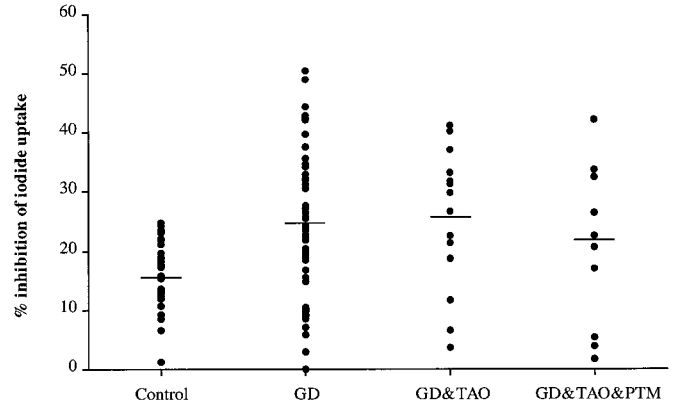

FIG. 5. The inhibition of iodide uptake by normal sera (n = 31), GD patients (n = 64), GD patients with TAO (n = 14), and PTM (n = 10). Results are the mean of duplicate cultures and are expressed as percentage of inhibition of iodide uptake in CHO-NIS9.

TABLE 1. Inhibition of iodide uptake in CHO-NIS9 by sera from patients with GD

	Inhibitory	Noninhibitory	% Inhibitory
GD sera (n = 88)	27	61	31%
New GD sera (n = 26)	8	18	31%
TAO sera (without PTM; n = 14)	6	8	43%
PTM sera (n = 10)	3	7	30%

New GD sera represents previously untreated patients, TAO patients with associated ophthalmopathy, and PTM with associated pretibial myoedema. Significant iodide uptake inhibition is taken as any inhibition in excess of the mean + 3 SD of the results with the control sera.

ference between GD and Multinodular goiter patients ($P = 0.02$), whereas no difference was detected between control and MNG samples ($P > 0.05$). No difference in the frequency of iodide uptake inhibitory activity of GD patient sera was found when the subgroups (*i.e.* newly diagnosed cases, GD with TAO or with TAO and PTM) were compared ($P > 0.05$). In addition, no correlation was detected between iodide uptake inhibitory activity and thyroid hormone levels or antithyroid peroxidase antibody titers in GD patient sera ($P > 0.05$).

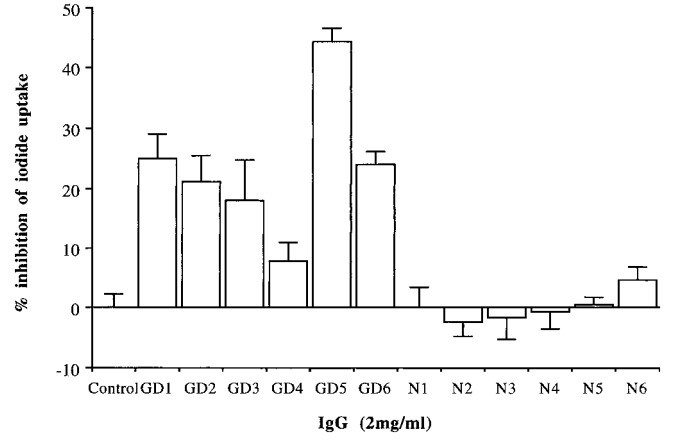

FIG. 6. The inhibition of iodide uptake by IgGs from GD patients (n = 6) and normal (N) controls (n = 6). Control represents the mean of four wells with added PBS without any IgG. Results are the mean of duplicate culture ± SEM and are expressed as percentage of inhibition of iodide uptake in CHO-NIS9.

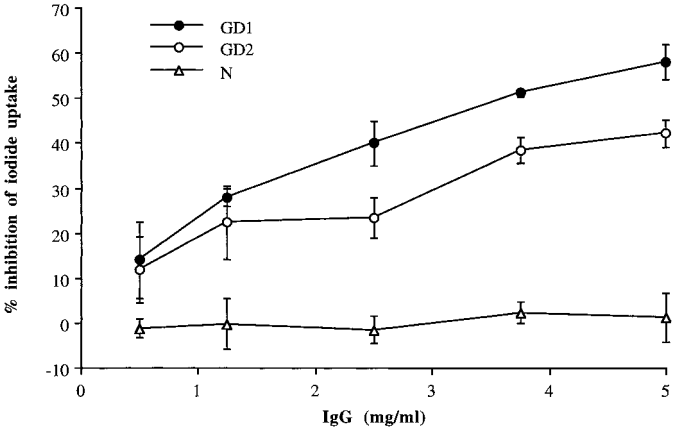

FIG. 7. The inhibition of iodide uptake by increasing concentrations of IgGs from two GD patients and one normal (N) control. Results are the mean of duplicate cultures ± SEM and are expressed as percentage of inhibition of iodide uptake in CHO-NIS9.

Discussion

In the present study, we have analyzed the basic characteristics of hNIS by expressing it in CHO-K1 cells. In agreement with work on rNIS, both perchlorate and thiocyanate competitively inhibited iodide uptake in CHO-NIS9 in a dose-dependent manner (9). Ouabain also inhibited iodide uptake by up to 85%, confirming the sodium dependency of iodide uptake in these cells. The time course for iodide uptake was relatively similar to that of the rNIS, reaching a peak after 30 min (9). However, half-maximal levels were reached after approximately 10 min, compared with 5 min documented for the rNIS (9). Iodide efflux studies showed that half of the radioactive iodide was released after approximately 10 min, which differs slightly from that observed for the rNIS (~15 min) (9). The above findings indicate that the physiological properties of hNIS, transfected into CHO cells, are largely similar to those of rNIS. Minor differences could be attributed to the different structure of the two molecules, although it is also possible that differential expression in the two CHO lines accounts for this variability.

The CHO-NIS9 cell line provides a tool for the detection of antibodies that modulate hNIS activity in patients with ATD. In this respect, it is difficult to test thyroid cell lines or primary thyroid cell cultures in bioassays for the presence of such antibodies, because other agents [including TSH and thyroid-stimulating antibodies (TSAb)] counteract their effects, rendering interpretation of the results difficult. One study has already analyzed the effects of ATD sera on rNIS activity (8), but as well as the xenogenic antigen used, only patients with HT were investigated. Therefore, we have studied the effects of GD sera on hNIS activity using the CHO-NIS9 cell line. Use of serum rather than Igs makes the resulting assay simple and rapid. Normal sera were used to establish whether these exert any inhibitory activity on iodide uptake by the transfected cells. The mean inhibition produced by normal sera was less than 20% (with maximum inhibition of 25%). This differs substantially from the study of Endo *et al.* (8), who documented more than 90% inhibition of iodide uptake by normal sera in CHO cells transfected with the rNIS. These contradictory results are possibly because of the different methodologies applied in the two studies, or could simply reflect the presence of nonspecific inhibitors in human serum against the rat but not hNIS.

Previous studies have shown that approximately 15% of HT sera bound to recombinant rNIS in Western blotting, and inhibited iodide uptake in CHO cells transfected with the rNIS (7, 8). On the other hand, 84% of GD sera reacted with the recombinant rNIS in Western blotting (7). However, the iodide uptake inhibitory activity of these sera was not tested. In the present study, 31% of GD sera inhibited iodide uptake in CHO cells transfected with the hNIS, and this was shown to be antibody mediated. Taken together, these results indicate that antibodies against the NIS, both binding and inhibitory, are more frequent in GD than HT patients. In addition, antibodies that react with the NIS may not necessarily modulate its activity, at least in patients with GD, but confirmation of this is needed in Western blotting experiments using hNIS. Extracts from CHO-NIS9 cells do not produce antibody binding in such experiments, which may relate to antigen concentration or the absence of linear determinants within the hNIS. The finding of NIS antibodies in 6.5% of MNG patients is compatible with the concurrence of other thyroid antibodies in some of these patients (13).

These results have several clinical implications. Antibodies that inhibit the function of NIS may partially counter the effect of TSAb in GD. This would explain the lack of correlation between the level of TSAb (or NIS antibodies) and the severity of clinical hyperthyroidism in some patients with GD (14). Antibodies against the symporter may also contribute to hypothyroidism in HT, at least in the initial phases of the disease, before widespread tissue destruction takes place.

The rapid and simple assay we have established with CHO cells transfected with hNIS should allow a detailed study of the role of the symporter as an autoantigen in thyroid autoimmunity.

Acknowledgments

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