

Isolation, banking, encapsulation and transplantation of different types of Langerhans islets*

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KEY WORDS

artificial pancreas,
cryopreservation,
experimental
diabetes,
immunoisolation,
islet transplantation

ABSTRACT

INTRODUCTION The discovery of a cure for diabetes is a dream of many medical researchers. The transplantation of Langerhans islets is a potential treatment of choice for patients with type 1 diabetes as a source of endogenous insulin for the recipient.

OBJECTIVES The aim of the experiment was to transplant Langerhans islets without immunosuppression. To protect the grafts against transplant rejection, semipermeable membranes could be used.

MATERIAL AND METHODS Langerhans islets were isolated from rats and pigs and immunoisolated by encapsulation in alginate-protamine-heparin (APH) or alginate-poly-L-lysine-alginate (APA) membranes. Islets were pooled in a controlled manner. Tests for cryopreservation and biocompatibility were also performed.

RESULTS The capsules coated with APH are more resistant than the capsules coated with APA. After transplantation of the islets immunoisolated with APA, euglycemia is maintained longer than after transplantation of the islets immunoisolated with APH. Microencapsulation protects the islets from destruction by the host.

CONCLUSIONS It is feasible to treat experimental diabetes by transplantation of encapsulated Langerhans islets without immunosuppression.

*Imagination is more important than knowledge.
Knowledge is limited.*

Imagination encircles the world.

*However... Truth is what stands the test
of experience.*

— ALBERT EINSTEIN

INTRODUCTION Diabetes mellitus is a metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion or insulin action, or both. This is one of the most prevalent, costly, and debilitating diseases in the world. Although conventional insulin therapy has alleviated the short-term effects, long-term complications are ubiquitous and harmful. Chronic hyperglycemia is associated with long-term

damage, dysfunction and failure of various organs including retinopathy with potential loss of vision, nephropathy leading to renal failure, peripheral neuropathy with a risk of foot ulcers, amputation and Charcot's arthropathy. Patients with diabetes have an increased incidence of atherosclerotic cardiovascular disease.

Transplantation of pancreatic islets might be the treatment of choice for patients with type 1 diabetes as a source of endogenous insulin for the recipient.¹ Grafted islets ensure strict control of glycemia and prevent hyperglycemia which may cause the above-mentioned, fatal complications. In clinical trials partial regression of retinopathy and neuropathy after the pancreas or Langerhans islet transplantation has been observed, but both

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Received: February 18, 2009.
Accepted: February 23, 2009.
Conflict of interest: none declared.
Pol Arch Med Wewn. 2009;
119 (5): 311-316
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Kraków 2009

* The study is dedicated
to the memory of
Professor Tadeusz Orłowski.

of these methods find clinical application only in individual cases.² Moreover, to protect the allografts or xenografts against transplant rejection, strong immunosuppressive treatment or, preferably, graft immunoisolation are required.

A bioartificial pancreas^{3,4} may become a promising approach to prevent or reverse complications associated with diabetes. Bioartificial pancreatic constructs are based on islet encapsulation, which involves using an artificial membrane to protect transplanted tissue from the host immune system. Semipermeable membranes used for transplant immunoprotection must be biocompatible, nondegradable, selectively permeable and resistant to mechanical stress. They should have appropriate diffusion properties for oxygen, nutrients, glucose and cell metabolic products, and should be impermeable to both the cellular and humoral components of the host immune system. This method eliminates the need for immunosuppressive drugs, and offers a possible solution to the shortage of donors as it may allow the use of animal islets or insulin-producing cells engineered from stem cells. Extravascular approaches are either macroencapsulation^{5,6} (large numbers of islets together in one device, hydrogel matrix or capillary) or microencapsulation^{7,8} (envelope for each individual islet in a semipermeable immunoprotective capsule). The microencapsulation approach is quite promising because it shortens the way of diffusion and the transplantation is technically easier than in macroencapsulation.

Both patients and physicians hope for the possibility to effectively cure insulin-dependent diabetes mellitus. It was also the great ambition of Professor Tadeusz Orłowski. In 1982, Professor Orłowski was appointed the head of the laboratory at the Transplantation Institute of the Medical University of Warsaw and the Institute of Biocybernetics and Biomedical Engineering (IBBE) at the Polish Academy of Sciences.

The laboratory has conducted research on the methods of Langerhans islet isolation from animals including mice, rats, and pigs, and initially also from human pancreas. A range of procedures for purification of the tissue have been used. Different methods of islet immunoisolation have been applied and the effect of encapsulated islets on the host immune system has been investigated. Another area of research has been the cryopreservation of islets to collect a sufficient number of proper islets for the transplantation to be successful.

MATERIAL AND METHODS Obtaining good islet isolation is one of the most important factors behind the success of islet transplantation. A successful method of islet isolation was initiated in 1965 by Moskalewski,⁹ who suggested the use of collagenase solution for pancreas digestion.

Rat islet isolation Initially, various procedures of islet isolation were applied including mechanical crumbling of the pancreas, multiple digesting

in collagenase solution range, or an introduction of collagenase solution to the pancreas after resection.^{10,11} In 1967, Lacy and Kostianowski further developed the method of islet isolation by introducing collagenase solution to the common bile duct before pancreas resection.¹² This method, with some modifications, has been used for pancreas digestion and islet isolation until now.

In our laboratory we use the infusion to common bile duct 1000 U/ml concentration of collagenase (Boehringer P) and pancreas digestion at 37°C for 10–13 minutes.

Pigs' islet isolation We harvested >300 pancreases from market slaughterhouses, from pigs weighing 100–250 kg. In the slaughterhouses, the pancreases were dissected *ex situ* immediately after killing and subjected to exsanguination, water (60°C) bathing, and skin shaving (warm ischemia time 15–30 min.). The main pancreatic duct was cannulated and infused with digestion medium (depending on the method) at 4°C. The organ was transported (4°C, cold ischemia time >190 min) to the laboratory. Initially, five different methods of pancreas digestion were used (Ricordi,^{13,14} Horaguchi,¹⁵ Ricordi and Horaguchi, Viviani,¹⁶ our original method¹⁷). Currently, we use our own solution for islet isolation: pancreas digestion with digestion medium in the plastic bottle with or without minimal shaking. Digestion is terminated when free islets are detected under the microscope. After filtration across 300-μm mesh, the islets are washed, suspended in Hanks's solution, and quantitated. Their number is converted into 150-μm diameter islet equivalents (IE). Islets of <50 μm in diameter are not counted. The viability of islets is confirmed by dithizone staining.

Microencapsulation The aim of our study was to find a practically applicable method to reduce the barrier between encapsulated islets and the bloodstream in order to improve both the functional performance and the survival of encapsulated islet grafts. Microencapsulation was proposed by Sun¹⁸ and has been the most popular and modified encapsulation method to date. The idea behind this method is to enclose one islet in a semipermeable membrane. Alginate is the most common and one of the most promising biomaterials used for encapsulation of allogeneic and xenogeneic cells and tissues (e.g. Langerhans islets). In the alginate system, the process of microcapsule formation involves the gelation of an alginate-cell suspension in a calcium chloride bath. Droplets are made using our own impulsed voltage droplet generator designed at the IBBE.¹⁹ Briefly, handpicked islets are suspended in 1 ml of 1.5% (wt/vol) sodium alginate (Sigma, St. Louis, MO, USA) and the droplets formed by the droplet generator are gelled in 1.1% CaCl₂ solution. To improve the strength of alginate capsules and create a better immunoprotective barrier, several layers of capsule membranes are applied:

TABLE 1 Response of microcapsules to different kinds of mechanical and chemical stress²⁶

Kind of stress	Type of capsules	
	empty alginate capsules coated with protamine-heparin membrane	empty alginate capsules coated with poly-L-lysine-alginate membrane
suspension in distilled water	some swelling	got wrinkles
suspension in 1N HCl [pH = 3.54]	resistant	resistant
suspension in >0,003 N NaOH [pH = 8.4]	resistant	resistant
suspension in >0,025 N NaOH [pH = 10.84]	resistant	dissolved
cryopreservation	resistant	got wrinkles
ultrasonic homogenization with "Labsonic U" power	to third attempt	after first attempt
repeating duty maximal	resistant	destroyed
repeating duty cycle 0.9 of 6' each	resistant	destroyed

1 alginate-protamine-heparin complex (APH) according to Tatarkiewicz.^{20,21} alginate capsules are exposed successively to 0.25% polyethyleneimine solution, 0.1% protamine sulfate solution, and 25 U/ml heparin solution.

2 alginate-poly-L-lysine-alginate (APA) according to Sun.²² alginate capsules are exposed successively to 0.05% poly L-lysine (Sigma) and to 0.15% sodium alginate. Finally, alginate gel inside microcapsules is liquefied by 0.05 molar sodium citrate. Thus formed microcapsules are spherical and smooth, with reproducible diameters of 300–500 microns.

Biocompatibility and resistance of APH and APA membranes Empty capsules were transplanted into rats under skin, intraperitoneally and under kidney capsule and were observed to be generally harmless at 1, 2, 3 and 4 months. Histological evaluation performed after this time^{23,24} demonstrated that they did not adhere to the neighboring tissues and were not covered with any fibrous tissue for the both membrane types investigated. The empty capsules were tested on resistance by ultrasonic damage, alkalization, several passages through syringe needles, osmotic stress, and freezing.²⁵

Cryopreservation The effectiveness of Langerhans islet transplantation in humans is limited due to difficulties in isolating a sufficient number of islets from a single donor. Cryopreservation enables to collect a sufficient number of islets for transplantation. Islets isolated from the pancreas, non-encapsulated (free – F) or encapsulated (E), were cryopreserved using the Rajotte^{26,27} method of slow cooling and rapid thawing.

The effect of xenotransplantation of encapsulated islets on the host immune system We evaluated whether xenotransplantation of rat islets encapsulated by APA membrane to mice would specifically stimulate the host immune system and verified membrane performance in a system with encapsulated material capable of specific stimulation. The first (control) group of the recipients were naïve. The second group of the recipients were immunized *in vivo* by intraperitoneal

transplantations of 500 non-encapsulated (free islands – FI) or 500 encapsulated by APA membrane islands (E_{APA}I) and 500 APA capsules. Ten to fourteen days after grafting from immunized mice the splenocytes were obtained for *in vitro* examination. To observe the immunogenicity of the transplanted material *in vitro*, a modified classic culture used for studying immunore-sponse, the one-way mixed splenocyte and islet culture (MSIC) test, was used.^{28,29}

The spleen cells obtained from naïve or xenotransplanted recipients were used as responder splenocytes in the test. The research material was divided into the following groups:

1 splenocytes received from non-sensitized mice stimulated in test by free islets (C_i), encapsulated islets (C_{EI}) or empty capsules (C_{APA}),

2 splenocytes isolated from mice immunized by non-encapsulated islets (FI) and encapsulated islets (E_{APA}I) and empty capsules (C_{APA}).^{30,31}

MSIC test preparation: as stimulators 20 islets or 5 × 10⁵ splenocytes (non-encapsulated or encapsulated), pretreated with mitomycin C (5 µg/ml; Sigma), were co-cultured together with responders 5 × 10⁵ splenocytes prepared from naïve and immunized mice, for 1–5 days at 37°C in 95% humidified atmosphere of 5% CO₂. After 1–5 days of culture [³H] thymidine (0.5 µCi per sample; Świerk, Poland) was added and cells were harvested after 24 hours. Samples were counted in a scintillation counter. Proliferation of responder splenocytes as response to antigen stimulation was expressed as Stimulation Indices (SI) and calculated as (count per minute [cpm] of responder splenocytes + stimulator)–(cpm of stimulator alone) divided by (cpm of responder splenocytes alone).

Skin grafting To sensitize the immune host the skins were transplanted 30 days before or after non-encapsulated or encapsulated rat's WAG islets of Langerhans (TABLE 1) were transplanted, and skin graft rejection and glycemia were observed.³²

Skin grafting was performed using the method of Billingham and Medawar.³³ Full-thickness skin (1 × 1 cm) from the belly of a donor rat was grafted onto the right side of the thorax

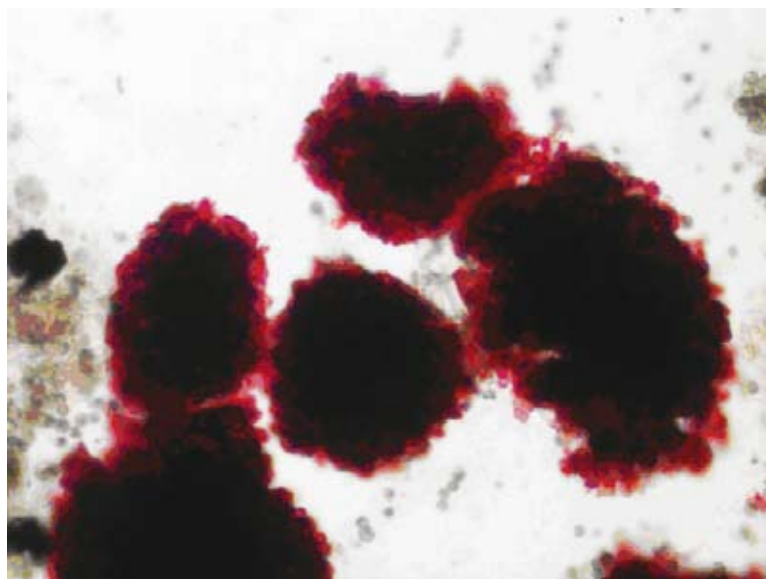


FIGURE 1 Pig islet diameter $>200\ \mu\text{m}$, dithizone staining (magnification of $40\times$)

of a recipient mouse. The graft was covered with gauze and plaster that were removed on day 5. Graft were scored daily until rejection (defined as loss of $>80\%$ of the graft tissue).

The length of islet graft survival served as a criterion for the sensitizing capacity of the primary graft and membrane protection. According to the design of the performed experiments, mice were classified into the following groups consisting of 7 animals each:

$D_F S$ = Skin (S) after non-encapsulated (F) islets transplantation; $DE_{APA} S$ = Skin (S) after encapsulated (E_{APA}) islets transplantation; $DSSI_F$ = non-encapsulated islet transplantation after the second skin transplant; $DSSE_{APA}$ = encapsulated islet transplantation after the second skin transplants.

Xenotransplantation of encapsulated Langerhans islets One thousand islets: free (group D_F), encapsulated with APA (group DE_{APA}) or encapsulated with APH (group DE_{APH}), were transplanted intraperitoneally onto streptozotocin-induced (intravenously $165\ \text{mg/kg}$ body weight) diabetic BALB/c mice. Only mice with 2 consecutive blood glucose levels $>350\ \text{mg/dl}$ were transplanted. Diabetic mice were used as a control (group D).³⁴

RESULTS Pig's islet isolation In all specimens, cell groups with diameters $<100\ \mu\text{m}$ contained

the smallest number of islets. Acceptable results (islet and/or IE number $>1000/\text{g}$; **FIGURE 1**) were obtained from only 56 of 348 slaughterhouse pigs, namely, 2073 ± 137.4 standard error (SE) (median, $1767/\text{g}$) islets. Most of them were big ($IE\ 2994 \pm 303\ \text{SE}$; median, $1874/\text{g}$). We have confirmed the great variability of islet yield, not only between particular strains but also between animals belonging to the same race. We identified slaughterhouses as the best source of pigs available in Poland. The poor yield obtained in our study probably resulted from the method of slaughtering used, namely a hot water bath. The main reason was the prevalence of little islets (80%) in digested organs in pig pancreas, which had already been observed by other researchers before.³⁵

Cryopreservation After 2–454 days of banking in a frozen state, the islets were thawed. The number of viable islets stained with dithizone, and the yield of thawed islets as a percentage of their pre-cryopreservation number were compared.³⁶ The yield of the thawed encapsulated islets was higher than that of the non-encapsulated islets ($E = 83\% \pm 1.3\ \text{SE}$; $F = 65\% \pm 8\ \text{SE}$). The thawed encapsulated islets demonstrated better survival: $E = 90\% \pm 3.5\ \text{SE}$ stained with dithizone; $F = 70\% \pm 5.1\ \text{SE}$.

Microencapsulation Several features were comparable in the APH and APA capsules. Both the APH (**FIGURE 2A**) and APA (**FIGURE 2B**) capsules were spherical and smooth, with reproducible diameters of approximately $400\ \mu\text{m}$. Empty APH capsules were stronger than APA (**TABLE 2**). The walls of both capsule types displayed selective permeability.

Intraperitoneal transplantation into streptozotocin-diabetic mice of 1000 non-encapsulated rat islets restored normoglycemia for 5–9 days (DI_F). Immunoisolation of islets by encapsulation before transplantation with APH membrane can reverse hyperglycemia for >6 weeks (46 ± 15 days), whereas APA-isolated grafts can secrete appropriate amounts of insulin much longer, i.e. more than 180 days (**TABLE 3**).

In vivo, both APH and APA capsules grafted into the peritoneal cavity or under renal capsule provoke no inflammatory reactions and are free from cellular and fibrotic overgrowth.

FIGURE 2 **A** – empty APH capsules (magnification of $4\times$), **B** – empty APA capsules (magnification of $4\times$)

Abbreviations: APA – alginate-poly-L-lysine-alginate, APH – alginate-protamine-heparin

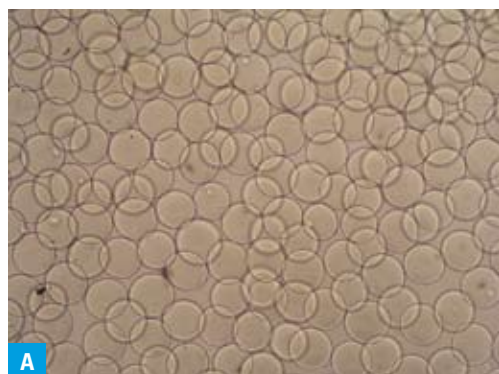


TABLE 2 Reversal of hyperglycemia after islet xenotransplantation into diabetic mice

Group	n	Kind of transplanted islets	Glycemia before transplantation	Glycemia after transplantation	Normoglycemia time
			mg%	mg%	[days (SD)]
D	7	non-transplant	>400	>400	0
D _F	7	free islets	>300	100–200	6.1 (2.0)
DE _{APH}	7	islets coated with APH membrane	>350	120–250	46 (15)
DE _{APA}	7	islets coated with APA membrane	>400	120–200	162 (85.4)

Abbreviations: APA – alginate-poly-L-lysine-alginate, APH – alginate-protamine-heparin

D – diabetic, E – encapsulated, F – non-encapsulated islets, SD – standard deviation

TABLE 3 Effect of the immune system activity on glycemia in grafted BALB/c diabetic mice

Group	n	Transplantation protocol	First set survival time	Second set survival time	Third set survival time
			[days (SD)]	[days (SD)]	[days (SD)]
DE _{APA} S	7	Skin after encapsulated [APA] islets transplantation	>55 ^a	5.7 (0.2)	
DSSI _F	6	Free islet transplantation after 2nd skin transplant	8.5 (0.6)	7.16 (0.4)	0
DSSE _{APA}	8	Encapsulated islet transplantation the 2nd skin	8.3 (0.5)	7.2 (0.4)	2.7 (1.1)

a all mice euglycemic until death

Abbreviations: I – islets, S – skin, DES – skin after encapsulated islet transplantation, DSSI – non-encapsulated islet transplantation after two skin transplants, DSSE – encapsulated islet transplantation after two skin transplants, others – see **TABLE 2**

TABLE 4 Results of one-way modified MSIC test in particular groups. Stimulation indices

Groups	n	Stimulator in the MSIC test	Specific stimulation index (SD)
C _{FI} – non-immunized	7	free rat islets	24.5 (4.6)
FI – immunized with free rat islets	7	free rat islets	7.1 (2.1)
C _{EI} – non-immunized	7	encapsulated rat islets	0.93 (0.18)
E _{APA} I – immunized with encapsulated rat islets	7	encapsulated rat islets	3.4 (0.7)
C _{APA} – non-immunized	7	APA empty capsules	1.59 (0.54)
APA – immunized with empty APA capsules	7	APA empty capsules	2.0 (0.5)

Abbreviations: C – control, others – see **TABLES 2** and **3**

Skin grafting Graft survival times are presented in **TABLE 3**. After islet transplantation (DE_{APA}S group) euglycemia was maintained during the whole period of observation (up to 55 days) even after stimulation of the host by skin grafting. In diabetic recipients strongly stimulated by skin grafts (groups DSSI_F and DSSE_{APA}), subsequent transplantation of encapsulated islets did not restore euglycemia despite encapsulation.³⁷

The influence of xenotransplantation of encapsulated islets on immune system of the host

Results are presented in **TABLE 4**. After stimulation by non-encapsulated rat islets, strong proliferation of responder splenocytes in the MSIC test was observed C_I: SI = 24.5 ± 4.6 standard deviation (SD). Splenocyte response to stimulation by encapsulated islets was weak C_{EI}: SI = 0.93 ± 0.18 SD. Contrary to expectations, after recipient immunization by non-encapsulated islets, the proliferation of responder splenocytes was lower than in the control group FI: SI = 7.1 ± 2.1 SD. Proliferation of responder splenocytes, obtained from mice immunized by encapsulated islets in the MSIC test, was higher than in the control group but lower than

in the non-encapsulated islet group SI (C_{EI}) < SI (E_{APA}I) < SI (FI). Islet encapsulation reduced their immunogenicity.

We didn't observe significant differences after stimulation of the responder splenocytes obtained from mice after immunization with empty capsules APA: SI = 2.0 ± 0.5 SD was comparable to the control group C_{APA}: SI = 1.59 ± 0.54 SD.

APH capsules are more resistant but long-term experiment indicates that immunoisolation with APA microcapsules is more effective than with APH microcapsules.

Microencapsulation sufficiently protected the grafted islets and the remission of diabetes was maintained. Independently of the reason for donor antigen leakage through normal or damaged capsular wall, encapsulated islet transplant may stimulate the recipient's immune response. Nevertheless, encapsulation sufficiently protects the graft from destruction.

The present results are consistent with the opinion that encapsulation does not protect the host immune system from stimulation but rather acts as “artificial immunoprivileged site” shielding the graft from destruction. Precryopreservative

microencapsulation of Langerhans islets improves their yield and function. In the case of free islets, sucrose should be added to cryopreservation for thawing procedure. Transplanted material after encapsulation only slightly influences the proliferation of the recipient splenocytes.

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Izolacje, bankowanie, opłaszczanie i przeszczepianie różnych typów trzustkowych wysp Langerhansa*

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SŁOWA KLUCZOWE

cukrzyca
doświadczalna,
immunoizolacja,
mrożenie,
przeszczepianie
wysp, sztuczna
trzustka

STRESZCZENIE

WPROWADZENIE Wyleczenie cukrzycy to marzenie wielu lekarzy. Przeszczepienie wysp Langerhansa jest potencjalną metodą leczenia cukrzycy typu I bez konieczności stosowania insuliny egzogennej.

CELE Celem tych eksperymentów jest zbadanie możliwości przeszczepienia wysp trzustkowych bez konieczności stosowania immunosupresji. W tym celu, aby ochronić przeszczep przed odrzuceniem, można zastosować półprzepuszczalną, trwałą i biozgodną membranę.

MATERIAŁ I METODY Wyspy pozyskiwano z trzustek szczurzych i świńskich. Immunoizolację wysp Langerhansa przeprowadzono przez zamknięcie wewnątrz alginianowych kapsułek i otoczenie ich półprzepuszczalną błoną alginianową/protaminowo-heparynową (APH) albo alginianową/poli-L-lizynową (APA). Bankowano wyspy w sposób kontrolowany. Przeprowadzono testy na trwałość i biozgodność kapsułek.

WYNIKI Kapsułki powlekane błoną APH są bardziej trwałe niż kapsułki APA. Po przeszczepieniu wysp Langerhansa immunoizolowanych błoną APA dochodzi do znormalizowania poziomu glikemii na dłużej niż po przeszczepieniu wysp otoczonych błoną APH. Membrana zastosowana do immunoizolacji chroni wyspy przed zniszczeniem przez układ immunologiczny biorcy.

WNIOSKI Możliwe jest wyleczenie cukrzycy doświadczalnej przez przeszczepienie opłaszczonych wysp Langerhansa bez konieczności zastosowania immunosupresji.

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Praca wpłynęła: 18.02.2009.
Przyjęta do druku: 23.02.2009.
Nie zgłoszono sprzeczności interesów.
Pol Arch Med Wewn. 2009; 119 (5): 311-316
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