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Original Article

Polymeric Approach to Adjuvant System in Antibody Production against Leishmaniasis Based on Hybridoma Technology

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Abstract

Background: Leishmaniasis is a zoonotic disease, which is one of the serious public health problems in the world. Nowadays, antibody production using hybridoma technology may be a correct approach in terms of sensitivity in the diagnosis of diseases such as leishmaniasis. The aim of this study was investigation of the effectiveness of different adjuvants on polyclonal antibody production against *L. tropica* based on hybridoma technique.

Methods: Accordingly, Freund's adjuvant (1956, *M. tuberculosis),* as a classic adjuvant in studies, was used comparatively with the non-toxic polymeric based Polyoxidonium adjuvant. All animal immunization procedures were conducted at Bezm-i Alem University Experimental Animal Research Center. The adjuvant response was tested both in the serum sample and in the antibodies produced by the hybridomas. The antibody titers were determined with ELISA.

Results: Freund's and Polyoxidonium (PO) group blood titer's increased approximately 5.5 fold compared to control after the 6th and 8th immunization. Hybridomas produced from mice immunized with PO adjuvant induced only antigen-specific antibody response and did not develop an immune response against the adjuvant.

Conclusion: Adjuvant selection is very important in terms of the specificity of antibody responses of cells produced in hybridoma technology. Therefore, PO is recommended as a new adjuvant system in this study.

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Introduction

Leishmaniasis is a zoonotic disease caused by infection with one of the more than 20 species of *Leishmania* protozoa (1-5). Overall, 350 million people, adults and children, in 90 countries are living under the threat of leishmaniasis. There are 12 million people infected according to WHO data and 2 million new cases occur each year in the world. *L. tropica* and *L. infantum* are the main protozoan species causing cutaneous leishmaniasis (6). The development of resistance to antimonial drugs in parasites and to insectisist in the vectors, failure to develop an effective vaccine and problems of diagnosis are leading to the expansion of this disease (3,4,7). Thus, different methods are used for the diagnosis of leishmaniasis. These methods include microscopy, culture, microculture (5), serological and molecular methods. The widespread methods rely on the clinical indication and direct visualization of the parasites with the aspiration biopsy. The serodiagnosis methods have been recognized as the more sensitive and minor invasive methods compared to classical methods (8-10). However, serological methods have high sensitivity to visceral leishmaniasis, whereas their sensitivity against cutaneouse leishmaniasis is low (11). Accordingly, the development of diagnostic methods based on hybridoma technology is very important in the diagnosis of leishmaniasis (12).

In recent years, polyclonal and monoclonal antibodies have been used in the diagnosis of parasitic diseases, with analytical and therapeutic uses. The rK39 kit, commercially produced and used in the diagnosis of visceral leishmaniasis has a higher sensitivity in patients (13). The basic principle of the rK39 kit is based on the detection of antibodies to *Leishmania* parasite in the sample. Therefore, this kit is less susceptible to cutaneous leishmaniasis characterized by low antibody titer. The greatest advantage of diagnostic kits based on hybridoma technology is that it is based on the presence of any antigenic molecule specific to the parasite, not the antibody in the sample.

Hybridoma research on leishmaniasis have been not enough on the road to diagnosis and designing the optimal hybridoma protocol produce for until now. In these studies, *Freund's* adjuvant was often examined too (14-19). Freund's adjuvant is different from other adjuvants because it is more toxic and not active enough (20,21). However, there has not been a study showing the changes in antibody production and survival of hybridomas obtained using Freund's adjuvant until now. On the other hand, polymeric adjuvants such as Polyoxidonium (PO) do not cause toxic effects on cells and significantly increase the immunogenicity of antigens (22,23).

In this study, the efficacy of polyclonal antibody produced against *L. tropica* using different adjuvants was investigated.

Materials and Methods

Preparation of parasitic antigen

The freeze-thaw procedure was used for the large-scale production of *L. tropica (EP39)* parasites (24). Accordingly, the pellet was incubated at -196 °C for 10 min, at 37 °C for 10 minX5 (25). It was then centrifuged at 10.000 rpm for 15 min and measured in UV.

Myeloma cell cultivation

P3-X63-Ag8.653 myeloma cell line was cultured with 20% FBS and 1% Pen-Strep for RPMI and treated with 20 ug 8-azaguanine/mL prior to the hybridoma protocol (26).

Experimental Animals

All animal immunization procedures were conducted at Bezm-i Alem University Exper-

imental Animal Research Center, after approval by the Istanbul Bezm-i Alem University Experimental Animals Ethics Committee (Ethical number: 2013.42).

The study was started with six female Balb/c mice (6 wk old) in each group according to the statistical calculation. A blood sample of approximately 0.5 mL was taken for tail titer testing before vaccination and used for the control group(1:50). First group was immunized intraperitoneally with 0.2 mL volume of a mixture of 100 µg *L. tropica* antigen and 250-µg PO adjuvant (27). Second group was immunized intraperitoneally with 0.2 mL volume of a 1:1 mixture of 100-µg *L. tropica* antigen and 100 µg Freund's incomplete adjuvant (14). The mice were vaccinated two weeks apart until the antibody response was approximately 5 times higher than the control. The tail blood was drawn 0.4 mL before each vaccination. In this process, 1 mouse was lost in both groups. The experiment was continued with 5 mice in each group until 8th immunization. Only parasitic antigen was used in both groups at the last booster dose.

Cell fusion and cloning

Firstly, 10 wk old Balb/c mouse was sacrificed to collect peritoneum macrophages as a feeder layer for hybridoma cells on plates with 10%FBS RPMI. The Balb/C mice with titers 5–fold higher than control were sacrificed in two groups and mouse spleen cells were harvested. The spleen cells fused with myeloma cell (10:1) by the addition of PEG1500 (Sigma-Aldrich®) in RPMI with 20% FBS mixture. After centrifugation (1250 rpm, 10 min), the cells were resuspended in HAT (hypoxantine-aminopterin-thymidine) supplemented RPMI with 20% FBS. Diluted cell solution was seeded on a plate and incubated for 10 d under 5%CO_2 (28). The antibodies level were determined by taking supernatant samples on the first 10th day. For the next 10 d, it was incubated with HT (hypoxanthine-thymidine) medium and antibody quantification was performed by taking samples from the supernatant of the hybridomas on the 20th day (28).

Enzyme-Linked Immunosorbent Assay (ELISA)

The *L. tropica* parasitic antigen, LPG and adjuvants were prepared separately in 0.05 M, 9ml carbonate coating solution (pH: 9.6) as 1 mL. 100 µL of each prepared sample was added to a well on 96-well plates and incubated overnight at 4 °C. After washing three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20. Plates were coated with 2% milk powder. Serum samples were diluted 1:50 with 2% PBS/Tween 20/Milk. After the rewash step, serum or supernatant samples were added and incubated at 37 °C for 1 hour. Alkaline phosphatase conjugated IgG antibody was diluted 1:1000 with PBS/Tween20 and 100µL was added to each well as secondary antibody and incubated for 1 hour at 37 °C. Then 10 mg of substrate (p-Nitrophenyl Phosphate, Sigma-Aldrich), 200 mL dissolved in prepared substrate buffer (0.02 g ZnCl2; 0.04 g MgCl2; 1.5 g Glycine, pH: 10.4). The prepared substrate solution (100 µL) was added to each well and incubated for 30 min at RT in the dark. It was then measured at 405 nm in ELISA (29, 30).

Isolation, Purification and Characterization of LPG

The basic glycoconjugate, lipophosphoglycans (LPGs), found on the surface membranes of *Leishmania* parasites, is a virulence factor for leishmaniasis (31). Lipophosphoglycan (LPG) isolation, purification and characterization were performed using chloroform-methanol extraction and sonification processes and executed as a stationary phase *L. tropica* pellet. In the purification step, an octyl sepharose column was used in the chromatography. Thin layer chromatography was used for detecting carbohydrate in fractions with silica-coated paper pieces. The phenol sulphuric acid method was performed to define the amount of LPG (32). Then characteriza-

tion analysis was completed with Gel Permeation Chromatography-Size Exclusion Chromatography (GPC-SEC) at Yildiz Technical University.

Statistical Analysis

GraphPad Prism 5 software was used for statistical analyses. The data was analyzed by one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests.

Results

Evaluation of Antibody Titers after Immunization

In blood serum from mice, antibody response to *L. tropica* antigens was significantly increased compared to control in both groups (Fig. 1). The first measured value of the control group was around 0.1-0.2. As a negative control, the ELISA protocol was applied without adding serum and these wells were measured at a value between 0.04 and 0.06. These values were subtracted from all the obtained values while making statistics. After the 4th immunization, antibody development against *L. tropica* and/or adjuvant was measured in the experimental groups. At least a 5-fold increase, which may be sufficient for the hybridoma technique, occurred at the 6th immunization in Freund's group mice. On the other hand, it was realized after the 8th immunization of the PO group mice. According to *L.tropica* antigen coating, it increased approximately 5.5-fold in both groups versus control (Fig. 1).

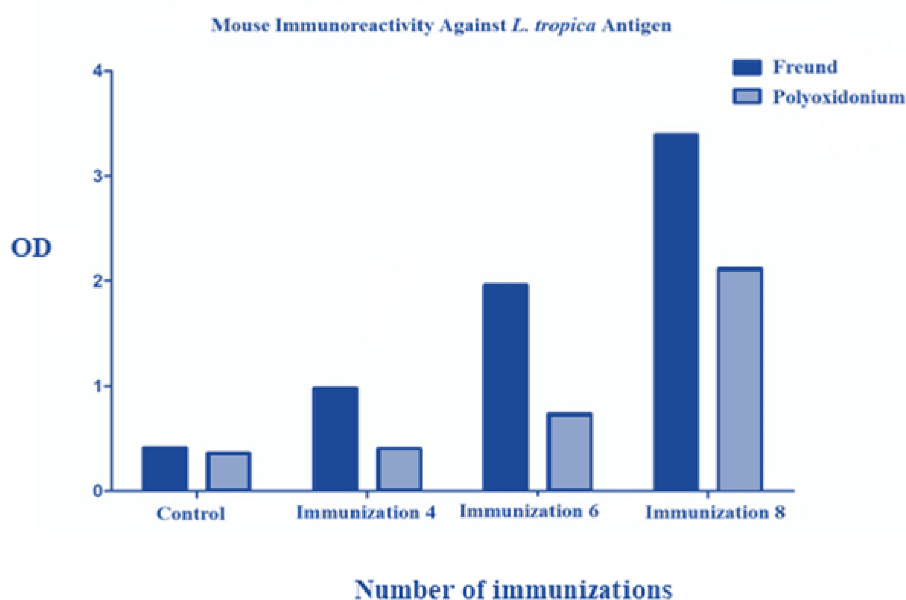

Fig. 1: Mouse immunoreactivity against *L. tropica* antigen. In mice developing specific for *L. tropica* parasite antigen, the antibody response increased 5.5 times in the 6th immunization in Freund's group and in the 8th immunization in the PO group

Then, the adjuvant coating was applied and the specificity of the same serum samples against the adjuvant was determined (Fig. 2). Serum responses in Freund's group also occurred against the adjuvant. The response was higher in the 8th immunization (Fig. 2b). In the PO group, there was no response to the adjuvant. It remained constant throughout the entire immunization period (Fig. 2a).

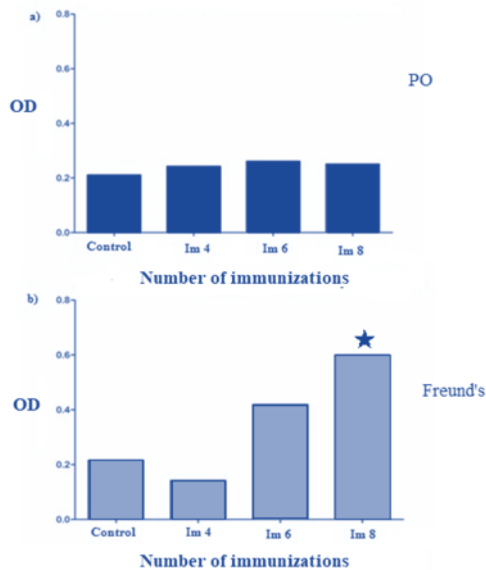

Fig. 2: Immunoreactivity after 4th 6th and 8th vaccinations with antigen and adjuvant complexes. Above) Immunoreactivity against PO adjuvant Down) Immunoreactivity against Freund's adjuvant. (Im: Immunization)

The responses in mice also improved against the adjuvant in *Freund's* group. In Freund's group, there was a statistically significant increase in the 8th immunization compared to the control (P<0.05). In the PO group, it was specific only to the antigen contained in the vaccine and there was no statistically significant increase or decrease. Accordingly, the response in Freund's group, used as a classical adjuvant, was not only against parasite antigens, but also against the adjuvant itself.

Morphological Analysis of Hybridoma Cells

Hybridoma cells were monitored for 30 days. From the 22nd day when the first colony was seen, images were taken on the 23rd, 25th and 27th d according to the colony growth (Fig. 3).

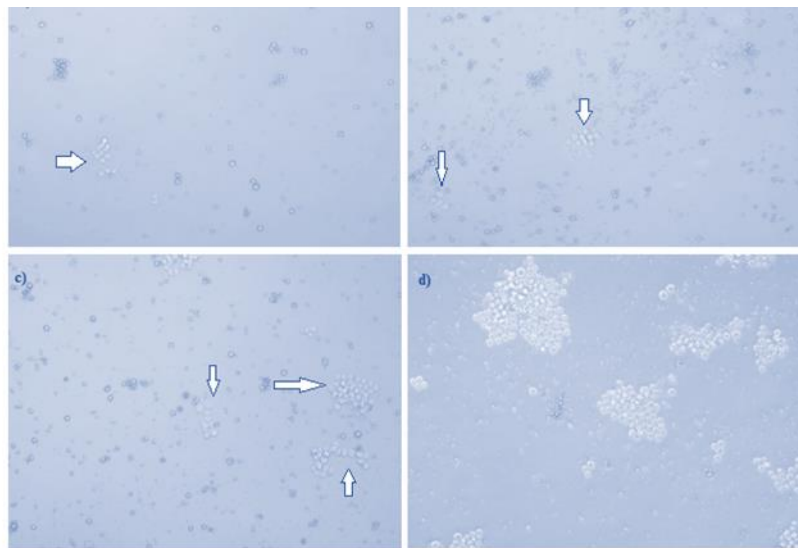

Fig. 3: Gradual development and colonization images of hybridoma cells a)22th b)23th c)25th d)27 th day. Morphological analysis shows a significant increase in the number of colonies in the PO group between the 22nd and 27th d of hybridomas. The increase in the number of hybridoma colonies was directly proportional to the increase in the amount of antibody produced against the antigen. On the other hand, in the Freund's group, no colony formation was observed following the formation of hybrid cells. This situation negatively affected the antibody response to the antigen

Antibody Levels Produced by Hybridoma Cells

The reactivity of the produced polyclonal antibodies against *L. tropica* antigen, PO and Freund's adjuvant was investigated. On the 10th and 20th d of hybridoma cultures, whether the activity of antibodies was only against the adjuvant was determined by ELISA by taking 50 µl of medium from the cell. The antigen-specific responses of Freund's group hybridoma cells were significantly reduced at day 20 compared to day 10 ($P<0.001$) (Fig. 4). The antibody response was reduced approximately 3-fold at day 20 of culture compared to day 10 in the Freund group. On day 20, an increase of approximately 2.5 times was observed in the PO group compared to the Freund's group (Fig. 4).

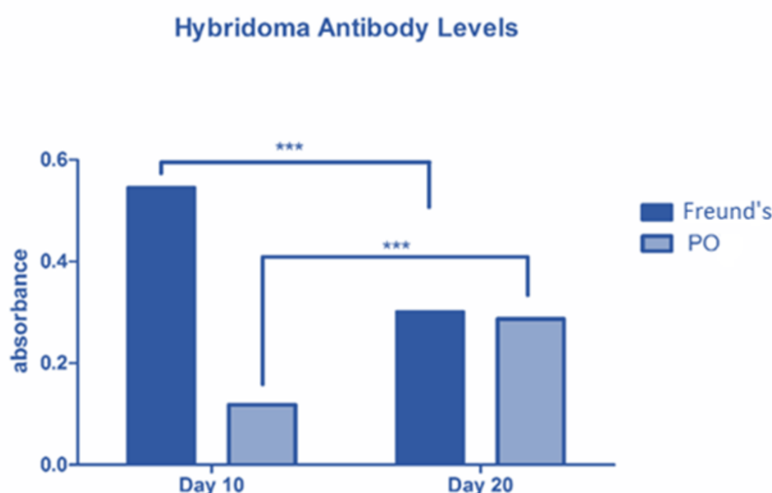

Fig. 4: Results of ELISA against *L. tropica* antigens 10th and 20th after fusion. While the response of hybridomas in the Freund's group decreased significantly; a significant increase was detected on the 20th day in the PO group.

In 10th and 20th the specificity of antibodies generated from hybrid cells to adjuvants was also measured. The antibody response of the hybridoma cells to PO adjuvant was significantly decreased by approximatly 4-fold until the 20th day ($P<0.001$). In addition, antibody levels produced by Freund's group of hybrid cells against *Freund's* adjuvant were significantly increased 20th approximatly 1.5-fold ($P<0.001$)(Fig. 5). The formed hybridoma colonies can develop both adjuvant and antigen specific responses.

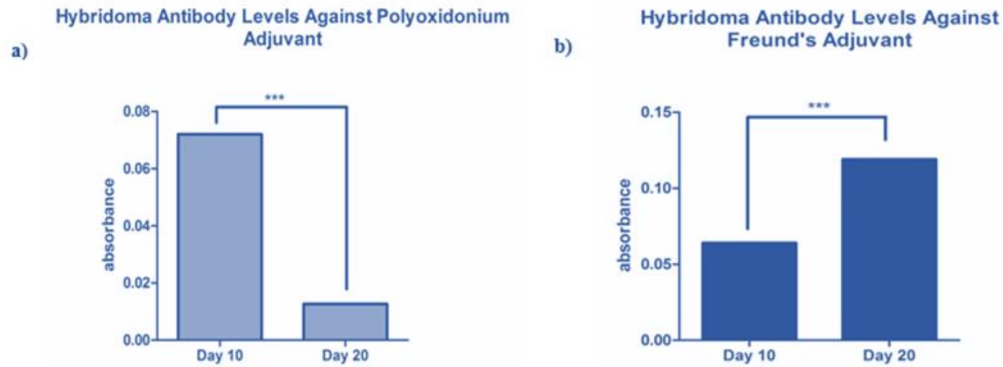

Fig. 5: Response of hybridoma cells agaist PO and Freund's adjuvants coating at 10th and 20th day after fusion. Hybridoma antibody levels against a)PO adjuvant b)Freund's adjuvant

While the response of hybrid cells to PO used as adjuvant decreases day by day, the response to the Freund's adjuvant was increased. On the other hand, the antibody responses to the parasite antigen of hybrid cells increased in the PO group and decreased in the other group. Sensitivity of the polyclonal antibody decreased against PO adjuvant but increased against the antigens.

Isolation and Characterization of Leishmania Surface Molecules (LPG)

It was analyzed by GPC-SEC for the characterization of LPG, the surface molecule obtained from *L. tropica* (Fig. 6). The chromatogram showed that LPG was eluted at 11 mL PBS. It was determined using the chromatogram data with the SEC method that the weight of the peak molecule is approximately 2000 kDa.

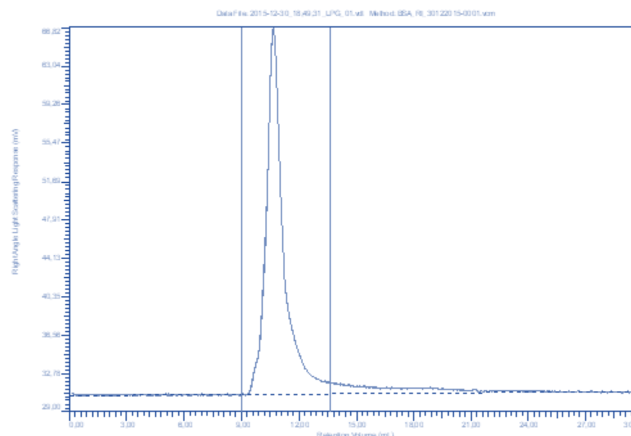

Fig. 6: GPC-SEC analysis of LPG. Chromatogram of isolation of LPG was obtained using coupling-light-scattering-size-exclusion-chromatography

Specificity of the Antibody Produced Against Different Leishmania Species and Leishmania Surface Molecule (LPG)

Antibodies produced by hybridomas were also examined against the LPG molecule isolated from *L. tropica* and against different *Leishmania* species (*L. infantum* and *L. major*). According to ELISA results, antibody specificity against LPG molecules was 60% higher than all parasite antigen. Polyclonal antibody

responses against different *Leishmania* species did not occur only in *L. tropica* (*P*<0.001) (Fig. 7). There was an antibody response due to the common structure of *Leishmania* parasite antigens. Hybridoma cells in this group showed the highest reactivity to LPG molecules isolated from *L. tropica*; the lowest reactivity occurred against *L. major* freeze-thawed antigens.

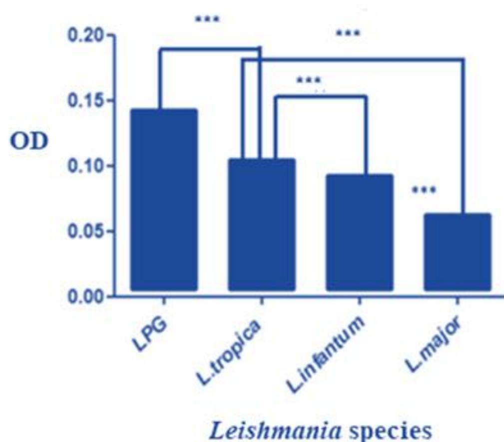

Fig. 7: Specificity of the produced antibody against *L. infantum, L.tropica, L.major*, antigen and *Leishmania* Surface Molecule LPG

Discussion

Diagnosis of leishmaniasis in laboratory is important because of its similar symptoms with diseases such as malaria, miliary tuberculosis, brucellosis, lymphoma and leukemia (32). The rK39 kit, commercially produced and used in the diagnosis of leishmaniasis, has a higher sensitivity in patients with visceral leishmaniasis (13, 33, 34). The greatest advantage of diagnostic kits based on hybridoma technology is based on the presence of any antigenic molecules specific to the parasite, not the antibody in the sample. This approach may help to overcome the serious problem of low sensitivity in blood samples of patients with cutaneous leishmaniasis.

In studies conducted in the literature, immunization was usually performed with live promastigote culture at the time of immunization and Freund's adjuvant was the standard adjuvant of choice (35-37). One of the main reasons for the inadequacy of studies on this field against leishmaniasis is the unsuitable adjuvant choice in generating an adequate immune response. Freund's adjuvant is a very important for TH1 and TH2 cell stimulation (38). However, storage at the injection sites, slow release of the antigen, low immunomodulatory effect and toxicity are the disadvantages of this adjuvant (19, 38). The toxic adjuvants may lead to problems with regard to affecting the life span of hybridomas and antibody production. We have not found any data in the literature about investigating how the adjuvants would affect the survival of hybridomas, production of antibody and other properties of the hybridoma cells to be generated.

In recent years, the studies on the use of more effective non-toxic adjuvants in vaccine technologies have been increasing as well. Polymeric adjuvant systems are highly preferred when it comes to increasing the 'site-specific degradation' potential of the vaccine (39, 40). In this study, we included polymeric-based adjuvant PO. We examined both the vaccine formulation created with the antigen and the transfer of the obtained antibody response to hybridoma technology and the effectiveness of the adjuvant in this process. Effective combination of PO (23) with frozen-thawed and autoclaved antigens has a high immunogenicity.

In this study, the comparison of Freund's adjuvant, frequently used in the literature, and PO as an alternative, was used for the first time in hybridoma technology. At the same time the effect of these adjuvants on antibody production have been examined besides. According to results, PO, which is a non-toxic, biocompatible, does not produce antigenic responses alone, has been acquired advantages ability of hybridoma cells to produce specific antibodies in a healthy manner. Last part of the study, the specificity of polyclonal anti-

bodies produced from hybridomas against LPG obtained from *L. tropica* and against all parasite antigens *L. major* and *L. infantum* were examined. This may indicate that the produced antibodies specific against like LPG molecule for importance surface part of *Leishmania* species. On the other hand, the specificity of polyclonal antibodies against all antigens prepared from other species was found to be lower. Since the LPG molecule exhibits interspecific polymorphism, LPG-specific polyclonal antibodies produce a lower response to species other than *L. tropica*.

Polymeric based adjuvants can be used in the production of antibodies that are based on hybridoma technology and contribute to the production of higher levels of antibodies compared to the conventional adjuvant applications and can lead to the formation of more healthy hybridoma colonies.

Conclusion

The vaccine-adjuvant relationship must include a controlled, feasibility, holistic approach that achieves the highest efficiency at the lowest cost. In addition to traditionally applied adjuvants, it is very important to develop new adjuvants that incorporate new technologies in the developing world and are more targetable, reduce the number of vaccinations, do not show immune response, but have a strong effect. Various adjuvant studies against different infections that may affect the whole world will shed light on science. In this context, polymeric-based PO, which examines its effectiveness on *Leishmania* parasite antigens and hybridoma antibody production as an alternative adjuvant candidate, was included in this study. Antibodies based on hybridoma technology to be developed against parasitic diseases are important biotechnological products with wide use in both diagnostic and therapeutic systems.

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Conflict of interest

The authors declare no potential conflicts of interest.

References

1. Roque AL, Jansen AM. Wild and synanthropic reservoirs of *Leishmania* species in the Americas. Int J Parasitol Parasites Wildl. 2014; 3:251-262.
2. Ertug S, Gultekin B, Doyuran ES. 2001 Yılında Aydın İl Sağlık Müdürlüğü'ne İhbar Edilen İç Organ ve Deri Leishmaniasis Olguları. ADÜ Tıp Fakultesi Dergisi. 2002; 3:9–12.
3. Handemir E, Oncel T, Kamburgil K. Istanbul Sokak Kopeklerinde Visseral Leishmaniasis Seroprevalansı. Turkiye Parazitol Derg. 2004; 28:123-125.
4. Organization WH. Expert Committee on the Control of Leishmaniases. In: Technical Report Series 2010; 949.
5. Serin SM, Dağlıoğlu K, Bağırova M, et al. Rapid diagnosis and genotyping of *Leishmania* isolates from cutaneous and visceral Leishmaniasis by microcapillary cultivation and polymerase chain reaction-restriction fragment length polymorphism of mini exon region. Diagn Microbiol Infect Dis. 2005; 53:209-214.
6. Ozbilgin A, Harman M, Karakus M, et al. Leishmaniasis in Turkey: Visceral and cutaneous leishmaniasis caused by *Leishmania donovani* in Turkey. Acta Trop. 2017; 173: 90-96.
7. Allahverdiyev AM, Uzun S, Bağırova M, Durdu M, Memişoğlu HR. A sensitive new micro-

culture method for diagnosis of cutaneous leishmaniasis. Am J Tro Med Hyg. 2004; 70: 294-297.
8. Harith AE, Kolk AH J, Kager PA, et al. Evaluation of a newly developed direct agglutination test (DAT) for serodiagnosis and sero-epidemiological studies of visceral leishmaniasis: comparison with IFAT and ELISA. Trans R Soc Trop Med Hyg. 1987; 81(4), 603-606.
9. Ashford DA, Badaro R, Eulalio C, et al. Studies on the control of visceral leishmaniasis: validation of the Falcon assay screening test-enzyme-linked immunosorbent assay (FAST-ELISA™) for field diagnosis of canine visceral leishmaniasis. Am J Trop Med Hyg. 1993; 48(1):1-8.
10. Ozensoy S, Ozbel Y, Turgay N, et al. Serodiagnosis and epidemiology of visceral leishmaniasis in Turkey. Am J Trop Med Hyg. 1998; 59(3): 363-369.
11. El Safi SH, Evans DA. A comparison of the direct agglutination test and enzyme-linked immunosorbent assay in the sero-diagnosis of Leishmaniasis in the Sudan. Trans R Soc Trop Med Hyg. 1989; 83: 334-337.
12. Miller J. "The thymus in immunity". Princ Med Biol. 1996; 6: 1-20.
13. Mathur P, Samantaray JC. The first probable case of platelet transfusion-transmitted visceral leishmaniasis. Transfus Med. 2004; 14(4), 319–321.
14. Khabiri AR, Bagheri F, Naddaf SR, Assmar M, Taghavi AH. Characterization of a Monoclonal Antibody Specific for the Parasite Surface Antigen-2 of *Leishmania major*. Iran J Public Health. 2004; 10-14.
15. Fong D, Chang KP. Surface antigenic change during differentiation of a parasitic protozoan, *Leishmania mexicana*: Identification by monoclonal antibodies. Proc Natl Acad Sci U S A. 1982; 79(23): 7366-7370.
16. Handman E, Hocking RE. Stage-specific, strain-specific, and cross-reactive antigens of *Leishmania* species identified by monoclonal antibodies. Infect Immun. 1982; 37(1), 28-33.
17. Nejad-Moghaddam A, Abolhassani M. Epitope mapping of a common 57 kDa antigen of *Leishmania* species by monoclonal antibodies. Vaccine. 2010; 28(37), 6036-6040.
18. Nejad-Moghaddam A, Abolhassani M. Production and characterization of monoclonal antibodies recognizing a common 57-kDa antigen of *Leishmania* species. Iran Biomed J. 2009; 13(4): 245-251.
19. Aguilar JC, Rodriguez EG. Vaccine adjuvants revisited. Vaccine. 2007; 25: 3752-3762.
20. Nouri A, Mohamed FAN, Laraba-Djebari F. New and safe formulation for scorpion immunotherapy: Comparative study between saponin and FCA adjuvants associated to attenuated venom. Vaccine. 2018; 36(13): 1720-1727.
21. Oscherwitz J, Hankenson FC, Yu F, Cease KB. Low-dose intraperitoneal *Freund's* adjuvant: Toxicity and immunogenicity in mice using an immunogen targeting amyloid-β peptide. Vaccine. 2006; 24(15): 3018-3025.
22. Dyakonova VA, Dambaeva SV, Pinegin BV, Khaitov RM. Study of interaction between the polyoxidonium immunomodulator and the human immune system cells. Int. Immunopharmacol. 2004; 4(13):1615-1623.
23. Toptygina A, Semikina E, Alioshkin V. Influence of an immunopotentiator polyoxidonium on cytokine profile and antibody production in children vaccinated with Priorix. Arch Physiol Biochem. 2012; 118(4): 197-203.
24. Aydogdu M, Bagirova M, Allahverdiyev A, et al. Large-scale cultivation of *Leishmania infantum* promastigotes in stirred bioreactor. J Vector Borne Dis. 2019;56(4):345-350.
25. Tosyali OA, Allahverdiyev A, Bagirova M, et al. Nano-co-delivery of lipophosphoglycan with soluble and autoclaved *Leishmania* antigens into PLGA nanoparticles: Evaluation of in vitro and in vivo immunostimulatory effects against visceral leishmaniasis. Mater Sci Eng C Mater Biol Appl. 2021; 120:111684.
26. Ethan A, Lerner BA. How to Make a Hybridoma. Yale J Biol Med. 1981; 54: 387-402.
27. Sereda AD, Makarov VV, Sachivkina NP, et al. Effectiveness of combined use: inactivated vaccines with immunostimulants on the *in vivo* model of teschen disease. Adv Anim Vet Sci. 2020; ISSN (Online) 2307-8316; ISSN (Print) 2309-3331.
28. Nourizadeh E, Zargar SJ, Alimohammadian MH, Ajdary S, Mahdavi M. Development of monoclonal antibodies against axenic amastigotes of *Leishmania infantum* strain in Iran: implication for diagnosis of Kala-azar. Iran J Basic Med Sci. 2018; 21(4):388.
29. El Roufaie Mohammed EA, Wright E, Kager PA, Laarman J, Pondman K. ELISA using in-

tact promastigotes for immunodiagnosis of kala-azar. Trans R Soc Trop Med Hyg. 1985; 79: 344-350.

30. Allahverdiyev AM, Koc RC, Bagirova M, et al. A new approach for development of vaccine against visceral leishmaniasis: Lipophosphoglycan and polyacrylic acid conjugates. Asian Pac J Trop Med. 2017; 10(9):877-886.
31. Topuzogullari M, Cakir Koc R, Dincer Isoglu S, et al. Conjugation, characterization and toxicity of lipophosphoglycan-polyacrylic acid conjugate for vaccination against leishmaniasis. J Biomed Sci. 2013; 3;20(1):35
32. Herwaldt BL. Leishmaniasis. Lancet. 1999; 354:1191-1199.
33. Rezaei Z, Van Reet N, Pouladfar G, et al. Expression of a rK39 homologue from an Iranian *Leishmania infantum* isolate in *Leishmania tarentolae* for serodiagnosis of visceral leishmaniasis. Parasit Vectors. 2019; 18;12(1):593.
34. Sanchez MCA, Celeste BJ, Lindoso JAL, et al. Performance of rK39-based immunochromatographic rapid diagnostic test for serodiagnosis of visceral leishmaniasis using whole blood, serum and oral fluid. PLoS One. 2020; 15(4):e0230610.
35. Paul WE. The Immune System: An Introduction, Fundamental Immunology. 7th Ed 2013.
36. Delves PJ, Martin SJ, Burton DR, Roitt IM. Roitt's essential immunology. Wiley-Blackwell. 2011.
37. Janeway CA, Travers P, Walport M, Shlomchik M. Immunobiology: The Immune System in Health & Disease. 2005.
38. Freund J. The mode of action of immunological adjuvants. Adv Tuberc Res. 1956; 7:130–148.
39. Basu P, Saha N, Saha T, Saha P. Polymeric hydrogel based systems for vaccine delivery: A review. Polymer. 2021; Volume 230, 124088.
40. W. Jianga, R.K. Gupta, M.C. Deshpande, S.P. Schwendeman. Biodegradable poly (lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens. Adv Drug Deliv Rev. 57. 2005; 391–410.